MANUAL OF

LABORATORY MEDICINE

THIRD EDITION

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TABLE OF CONTENTS

PREFACE

It came as a surprise that the second edition of Manual of Laboratory Medicine was completely exhausted in less than a year, despite the fact that it was printed in double quantity than the previous one. We were highly encouraged by this response. It appears that this manual has become an important part of every laboratory library in the country. There is news that some copies of the manual have been seen in the neighbouring countries as well.

Compilation of this manual is mainly the effort of the staff and senior postgraduate students of Armed Forces Institute of Pathology, Rawalpindi. It is thus, mainly based on practices and methods employed in AFIP and other Armed Forces hospitals. We are aware of the fact that the choice of methods varies from lab to lab based on the experience of its workers and availability of instruments and reagents. Therefore it still has a lot of room for improvement. We request not only the users of this manual but also our senior colleagues to make suggestions for further improvements in the text.

We have thought several times for including colour pictures where required but refrained because of the cost. This will make the Manual beyond the reach of many. We are however, seriously thinking to bring out a version on computer CD which can include colour pictures and at the same time the cost will not be too prohibitive.

Once again several changes have been made in this edition. Many older techniques have been deleted and several have been re-written to make them up to date. However, some old methods and techniques have been retained, which might seem unnecessary, but we feel would be of assistance to those who are working in minimally equipped laboratories, where fully automated procedures may not yet be available.

The contents have been updated and expanded but some material and basic framework from first and second editions has been retained.

Apart from the Editors and contributors listed, there have been several persons who contributed in typing and re-typing, shaping and printing of this manual. Available pages do not allow mentioning all of them. However we are greatly indebted to them for their valuable contribution.

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15 JUNE 2005

PREFACE TO FIRST EDITION

Laboratory medicine is key to practice of clinical medicine. It would be hard to imagine a situation where adequate medical care could be provided to the patients in the absence of comprehensive and reliable laboratory services. In the recent years it has become increasingly difficult for Specialist in Pathology and laboratory workers to keep pace with rapid developments in this field. Every day, new concept is being introduced. This position is very hard for a country like ours, where teaching opportunities are difficult to find especially in places out side the main urban centres.

A large number of publications providing comprehensive and up to date information are already available. However, most of them have been written abroad and are not related to the conditions, which prevail in our institutions. The laboratory workers in our country find it difficult to seek answers to the problems they face. This book has been written with a view to provide a comprehensive yet short account of laboratory procedures. The emphasis has been on the practical aspects of performing various tests and the associated pit-fall. A short account of the instruments and equipment employed has also been provided.

In a work like this, which endeavours to cover all the disciplines of pathology, it is not possible to comprehensively cover each and every test nor has there been any attempt to discuss in detail either the interpretation or the clinico-pathological background of these tests. As far as possible simple language has been used which our technicians with their limited educational background can also understand. It would be very useful for the laboratory workers manning a medium-sized laboratory.

A number of contributors are responsible for writing this book. Many of them have had vast experience of working and manning the laboratories. A significant proportion of young Specialist in Pathology who has personal experience of the difficulties, which are faced in small to medium sized laboratories, has also contributed. In addition, a large number of senior technicians have also offered very useful suggestions. We are grateful to them for their contribution.

In spite of the efforts, which have been involved in writing this manual, there are bound to be a number of omissions and deficiencies. Some of the omissions are deliberate and are designed to keep the book within limits of the stated objective. As regards deficiencies, we shall be grateful if these are communicated to us so that we cater for them in the next edition.

We are grateful to Gen Suhail Abbas Jafri, Surgeon General Pakistan Army for his encouragement and guidance without which it may not have been possible to undertake this work. We are also indebted to Lt Gen (Retd) S A Ahmad and Professor N A Jafry for their expert guidance.

We gratefully acknowledge the comments offered by Col Amir Hussain Khan, Lt Col Shabir Ahmed Kiani, Major Sajjad Hussain Mirza, Major Sajid Mushtaq, Major Muhammad Ashraf and Dr. Muhammad Tariq Khan, which were extremely useful in removing some important deficiencies and omissions.

Lastly, we acknowledge the secretarial assistance provided by Steno Muhammad Shafique, Hav Sarwar Khan, Hav Muhammad Rashid and the work of Mr Ashraf, our Artist and Mr Muhammad Saleem Baig our photographer in preparation of illustrations.

Manzoor Ahmad
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SECTION I - THE PATHOLOGY LABORATORY

1. ORGANISATION AND MANAGEMENT OF PATHOLOGY SERVICES

Pathology service in a hospital, is concerned with laboratory investigations of patients and at times with laboratory aspects of detection and prevention of disease. It includes a system of clinical advice or a request for the investigation, system for analyses of material received or collected and a system for interpretation of results and advice in a time scale relevant to the urgency of clinical problem. A complete service also includes the organisation of a chain; from specimen collection to receipt of the written report by the doctor in charge of the patient. All the functions are carried out in the designated area, the **Pathology Laboratory**, under the supervision of a **Pathologist**, who is also responsible for providing guidance to clinical colleagues on the best use of Pathology services. The consultant in each department is responsible for the report, which is issued. Pathology service depends on the coordinated activities of a number of professionals e.g., laboratory technicians, phlebotomists, biomedical engineers, electricians etc. The managerial responsibility for the performance of the service is usually placed on a senior consultant pathologist. The size and the complexity of the service will depend on the population in the community, bed strength of the hospital and type of clinical problems being dealt in the hospital.

FUNCTIONS OF A HOSPITAL LABORATORY

A hospital laboratory has to perform the following important functions:

1. To meet the requests for laboratory investigations by maintaining adequate diagnostic facilities.
2. To arrange for laboratory investigations from referral laboratories if not available in the premises.
3. To provide professional advise on management of the patient.
4. To monitor individual patients and provide laboratory control of therapy.
5. To provide laboratory facilities for research projects undertaken by clinicians.
6. To collaborate in the development, study and control of new methods of treatment.
7. To undertake applied research on pathology related problems.
8. To collaborate in education and training of medical and paramedical personnel.

ORGANISATION OF PATHOLOGY LABORATORY

Normally, a pathology laboratory will be allocated an area, proportional to its scope and load of work. This may then be organised into following units:

1. Administrative offices
2. Reception unit for registering patients, collection of pathology specimens from patients and delivery of reports.
3. Laboratory area organised into various sub-units. Normally, following major disciplines will be catered for:
   a. Haematology
   b. Chemical Pathology
   c. Microbiology
   d. Histopathology
   e. Medical supply stores
   f. Mortuary. This may be located away from the laboratory.

ROLE OF HEAD OF SERVICE

A senior consultant pathologist commonly heads the laboratory services. He is fully responsible for all the internal organisation and activities of the Pathology Laboratory as well as co-ordination with other departments, for provision of efficient laboratory services. To achieve these, he must have training and skills to analyse clinical demands and respond to them. His main duties include:

1. Provision of an efficient and cost-effective diagnostic and consultancy service.
2. Maintenance of performance standards including quality assurance.
3. Assurance of safety aspects in the laboratory including safety of employees.
4. Provision of scientific direction to the service including research and development.
5. Provision of or arranging for finances, personnel, equipment and accommodation for the services.
6. Assurance of effective use of available

resources.
7. Organisation of the training programme so that the work patterns are efficiently maintained.
8. To assign various work units and duties to the most suitable personnel available to him.

STRATEGIC PLANNING

Strategic planning is, although primarily the responsibility of the head of the laboratory but he must consult all the senior staff members. An analysis of the strengths and weaknesses as well as of ability to respond to opportunities and threats should be regularly performed. This is called SWOT analysis in business terminology. The results of these analyses should form the basis of future planning. Assessment of present and future workload is important for any planning exercise. There are several methods available for this purpose. One of these methods is the **Welcan System**. In this system one unit of workload corresponds to one min of productive time of technical and other staff involved. It includes the total time taken from receipt of the specimen or registration of patient to delivery of report.

COST ASSESSMENT

It is important to assess the cost incurred on the services provided, to adopt cost effective measures. If the workload has been properly assessed it is not difficult to assess the cost effectiveness by using following formula:

$$\frac{\text{Total laboratory Cost}}{\left[\begin{matrix}\text{No of}\\ \text{Operators}\end{matrix}\right]\times\left[\begin{matrix}\text{Welcan}\\ \text{units}\end{matrix}\right]\times\left[\begin{matrix}\text{Efficiency}\\ \text{index}\end{matrix}\right]}$$

Various methods of cost effective management have been developed and published. Detailed discussion of these is beyond the scope of this book. It is recommended that those Pathologists and Laboratory Technicians who are inspiring for key assignments in the laboratory services should make themselves conversant with these methods[1].

INDENTING AND STORAGE OF REAGENTS

A variety of reagents are used in the laboratory. Some are used almost daily and in large quantities while others are used less frequently. However, it is difficult to predict when and how frequently a reagent may be required. One of the important decisions to be made by incharge of any clinical laboratory is as to which reagent should always be present in the laboratory. While ordering or indenting a reagent following points must be considered:

1. **Shelf Life of Reagents**: Reagents with short shelf life should not be purchased in bulk otherwise a lot of them may be wasted. The manufacturer gives shelf life for every reagent. It may vary from a month (as for cell panels and haematology controls) to several years (as for most of the chemicals).
2. **Packed Quantity of Reagent**: Some reagents have a longer shelf life if kept in the original packing. But once opened or reconstituted, these have to be used in a very short time. Examples are reagents used in coagulation, immunological and serological tests. Sizes of packs of such reagents differ. One should select the size according to the requirements so as to prevent wastage.
3. **Storage Facilities in the Laboratory**: All reagents cannot be stored in ordinary cupboards or shelves. Some reagents, like inflammables require special area, some like poisons require safety cupboards, some can only be stored at 2-4°C, while others require deep freezers (-20 to -70°C) for storage. Thus, when ordering for any reagent, space available for that particular reagent must be kept in mind (see STORAGE OF LABORATORY REAGENTS on page 5).
4. **Quantity Required**: One must not order reagents at random because if these are not purchased in adequate quantity then one may face difficulty till next delivery. Whereas if purchased in excess, these may expire causing unnecessary loss. Quantity ordered should be carefully calculated. For calculating quantity of a particular reagent:
   a. Find out number of tests performed weekly and quantity of reagent used in each test.
   b. Calculate the quantity used weekly.
   c. Find out from records the percentage increase in the requests for that test over the last few years.
   d. Add to current requirement, the projected increase in consumption.
   e. Estimate losses of that reagent including wastage of reconstituted reagent, spillage, duplicate measurement, use in calibration or quality control etc. All these usually do not exceed 20%. Add this to previous calculation. This will be the net amount to be ordered.

      For example thromboplastin is to be ordered or indented for next one year.

[1] In preparation of this brief, help has been taken from various documents of UK NHS and Royal College of Pathologists on the subject, which is gratefully acknowledged.

This reagent is used in prothrombin time. Each test is done in duplicate and a control may be required for each test. Control test is also done in duplicate. Each test requires 0.2 ml of the reagent. Thus for each test 0.8 ml reagent {(0.2x2)+(0.2x2)} is required. If current workload is 8 tests per day then daily requirement is 0.8x8=6.4 ml. Suppose the daily workload was 4 tests a day, 4 years ago. It gives an average annual increase of 20%. Thus one may expect 20% increase in workload during next year. Therefore, one should add 20% (1.3 ml) to calculated amount. Similarly add another 20% (1.3 ml) for wastage. Thus net daily demand is 9 ml/day. From this one can calculate monthly, quarterly or annual demand.

5. **Frequency of supply**: One should consider shelf life of reagent and storage capacity for that reagent. In the above example of prothrombin time annual requirement is 9x365=3285 ml or 657 bottles of 5 ml. Shelf life of originally packed powdered reagent is on the average 3 months. Therefore, one cannot order more than 164 bottles each quarter. However, if allocated space is for only 11 boxes of 10 bottles each i.e., 110 bottles then requirement is met if one receives 11 boxes, every 2 months or so.
6. **When to order or indent**: Time point for ordering/indenting depends upon:
    a. Current stock position
    b. Time taken in processing of indent
    c. Time taken by supplier

It is always advisable to keep a substantial reserve to meet delays in supplies or increase in demand if shelf life of reagent permits. It may be convenient to place a standing order with instructions to the supplier regarding time of delivery. All such requirements can be programmed into a computer.

STORAGE OF LABORATORY REAGENTS

Many laboratory argents require special storage conditions. Improper storage may result in wastage or hazards like fire.

1. **Cold Storage**: A cold room or refrigerators and deep freezers are required for storage of most biological reagents, antisera, control organisms etc. Each reagent should be stored at temperature recommended by the manufacturer.
2. **Dark Store**: Many reagents are sensitive to light e.g., silver nitrate. These can be stored using dark (amber coloured) bottles or dark cupboards (interior painted black).
3. **Safety Cupboards**: These must be provided inside and outside the refrigerated room. These should have strong doors and good quality locks. All classified poisons must be kept in these. The stock record of each should be pasted or tied to the container. Keys should be deposited with a responsible person who should issue the required quantity when needed and make appropriate entry on the stock record (Bin card) and sign it.
4. **Inflammables**: These should preferably be stored at a distance. All such reagents have a flame mark on the label of the container. These should be kept in amber coloured bottles and storage area should be dark and cool. No flames or smoking should be allowed in that area. Electric wiring and fittings should be checked periodically to prevent any short-circuiting, which may cause fire.
5. **Acids and other Corrosives**: These should also be stored in specially allocated area. Bottles should be buried in sand to prevent spreading in case of breakage.
6. **Arrangement**: All stores should be maintained in some order for easy access. These can be grouped as mentioned above. In each group these should be arranged alphabetically for easy access.
7. **Stock Maintenance**: A proper stock register must be maintained. All additions or issues must immediately be recorded. Each bottle of any reagent in use must have card tied to it showing balance quantity in the bottle.

HAZARDS IN A PATHOLOGY LABORATORY AND SAFETY PRECAUTIONS

There are several types of potential hazards to be faced in a Pathology Laboratory. All the staff working in the laboratory must be fully aware of these, should make all possible effort to prevent these and should be prepared to face, if any of these occur. The hazards in a pathology laboratory mainly arise from:

1. Fault in construction of building and various installations.
2. Handling of infected specimens.
3. Handling of chemicals.
4. Faulty apparatus

These hazards can be broadly grouped into following five categories:

- Hazards to premises
- Hazards to environment
- Hazards to patients

- Hazards to staff
- Hazards to equipment

HAZARDS TO PREMISES

Like any other building, the premises of pathology laboratory are prone to hazards of **fire**. The chances are higher than an ordinary building because of multiplicity of electrical connections and use of flammable material. Preventive measures to be adopted include:

1. Insurance of electrification system of good quality and appropriate for the electrical load of the laboratory, installed under the supervision of a qualified engineer.
2. Timely replacement of any sparking socket.
3. Avoidance of use of temporary extensions and naked wires.
4. Safe and appropriate storage of flammable material.
5. Safe and appropriate storage of gases used in the laboratory.
6. Avoidance of unnecessary use of flammable items e.g., foam, wooden furniture, carpets etc.
7. Periodic training of staff for fire fighting.
8. Installation of fire alarm system.
9. Provisions of fire fighting equipment e.g., water hoses, fire extinguishers and sand etc., at suitable and appropriate intervals.
10. Display of telephone numbers of fire stations in the vicinity of the laboratory, in each room.
11. Provision of emergency Fire Exits and stairs.

In case the fire does occur, following should be done:

- Immediately call for help.
- Shut off the electric supply and gas supply.
- Evacuate any patients, women and children.
- Remove flammable material from near the site of fire.
- Fetch the nearest fire extinguisher or any other gadget of fire fighting and try to extinguish.

HAZARDS TO ENVIRONMENT

Hazards to the environment are often ignored. These arise from inappropriate disposal of laboratory waste which include:

1. Infectious material collected from the patients.
2. Used syringes
3. Poisonous chemicals
4. Radioactive material
5. Discarded tissue and organs
6. Polythene and latex material e.g., bags, gloves, gowns etc.

Following precautions should be taken to prevent environmental pollution

1. All infectious waste, which can be incinerated, should be carefully collected and burned.
2. All other infectious waste, e.g., urine, faeces, blood, fluids and cultures must be decontaminated before discharging into drainage system.
3. All syringes and needles should be cut into pieces to make them unusable and then destroyed.
4. All poisonous chemicals should be neutralised before discharging them into drainage system.
5. Radioactive waste should be collected in appropriate containers, allowed to decay and then disposed off according to regulations of Pakistan Atomic Energy Commission.
6. All left-over tissue/organs should either be cremated or buried deep in the soil.
7. Polythene and latex material should be decontaminated and preferably be recycled.

HAZARDS TO PATIENTS

The most important hazards to patients are:

1. Transmission of disease.
2. Vasovagal shock
3. Infection at the site of an invasive procedure.
4. Metabolic complication of some suppression or stimulation tests performed in endocrine disorders.

Following precautions should be taken to prevent these hazards.

1. Never use same syringe, needle, or canula of any type for two patients.
2. Non-disposable instruments, like bone marrow needles must be properly sterilised as per standard instructions. Still it is advisable to keep a separate set for patients known to be positive for hepatitis or HIV. This set should also be decontaminated in 0.5-1% Sodium hypochlorite solution for 10 min and then autoclaved.
3. All emergency medicines and equipment including that of cardio-pulmonary resuscitation (CPR) must be at hand where phlebotomy is done or other invasive procedures are performed to treat vasovagal shock.
4. All staff performing phlebotomy or other invasive procedures should be fully conversant with CPR procedures.
5. While performing an invasive procedure, including phlebotomy, the site should be

thoroughly disinfected with alcohol or a suitable iodine preparation.

6. The puncture site should be kept gently pressed to avoid any oozing and subcutaneous accumulation of blood to prevent infection.
7. The premises where stimulation or suppression tests are performed should be fully equipped to meet any emergency situation.

HAZARDS TO STAFF

Staff, particularly laboratory technicians, is most vulnerable to all the hazards. Blood, urine, faeces, CSF and other body fluids may contain highly infectious and potentially lethal organisms. These are collectively referred to as **biohazards**. Extreme caution is to be exercised while collecting, transporting, processing and disposing these off. All biologic specimens, regardless of the source, should be considered a biohazard. Following precautions must be observed:

1. Personal protective equipment e.g., gloves, mask, gown etc. must be worn when handling biologic specimens.
2. Practice of hand washing before and after dealing with biological material and patients should be inculcated in the staff.
3. No contaminated equipment or surface should be touched with bare hands.
4. Stoppers/lids from specimen containers should not be removed unnecessarily.
5. Mouth pipetting should never be allowed.
6. All non-disposable equipment should be frequently decontaminated.
7. It must be remembered that all unfixed and unstained slides are also infective.
8. All sharps, including needles and pieces of broken glass, must be handled with care and disposed off in cardboard containers.
9. All contaminated medical supplies should be decontaminated, autoclaved or incinerated.
10. All spills must be cleaned and surface disinfected immediately. Adopt following procedure:
    a. Protect yourself.
    b. Pick up sharps and glass pieces with forceps or pieces of cardboard.
    c. Clean the surface with household aqueous detergent.
    d. Disinfect with household bleach. Undiluted solution of good quality household bleach contains 5-25% sodium hypochlorite that is equal to 5000 mg/L of chlorine. For porous surfaces use 1:10 dilution of this solution but for hard surfaces 1:100 dilution is sufficient.
    e. Absorb with absorbent wool or paper towels.
    f. Rinse with water and allow drying.

HAZARDS TO EQUIPMENT

In modern laboratory most of the equipment is expensive and requires due care against any damage. There are three main sources of damage to the equipment:

1. Damage due to faulty electric supply.
2. Damage due to accumulation of corrosive material in various parts.
3. Damage due to rusting.

In our country the electric supply is not uniform. Not only the voltage fluctuates frequently but there are frequent shut down some times for a moment. This is a potential source of damage to all equipment requiring electric supply. Computerised equipment is particularly vulnerable. Following precautions should be taken.

1. All electric connections must be installed with good quality circuit breakers.
2. If possible, voltage stabilisers should be used. Circuit breakers should always be used with these.
3. Uninterrupted power supplies (UPS) should be used with computerised equipment to avoid repetition of tests, loss of data and damage to equipment.

Preventive maintenance of all equipment at regular intervals will safeguard against accumulation of corrosives and rusting. All technicians should be trained in this. This increases the life of the equipment.

SUMMARY OF SAFETY RULES

Good personal habits

- Use personal protective equipment
- Tie back long hairs
- Do not eat, drink or smoke in the work area
- Do not pipette with mouth
- Wash your hands before and after work

Good housekeeping practice

- Keep **work area** free of sharps, glassware and chemicals.
- Store every thing properly according to instructions by the manufacturer and safety regulations.
- Label all containers in bold.
- Paste warning signs at appropriate places.

Good laboratory technique

- Do not use unfamiliar equipment without proper learning.
- Do not perform any technique without proper learning.
- Read labels before using any reagent.
- Observe due precautions while transferring and mixing chemicals.

2. UNITS OF MEASUREMENT

Evolution of measuring systems closely parallels the evolution of civilisation. With increase in trade and communication between various parts of the world the necessity for a global or unified system of measuring became more and more obvious. For a long time two systems, namely the British and French systems of measurement, have been used in parallel to each other but then most of the world adopted French Metric system of measurements. History of metric system dates back to 1871 when **metre** was first introduced as unit of length. This unit was redefined in 1889. In 1863, in search for universally acceptable system, a system based on **metre** (as unit of length), **gram** (as unit of weight) and **second** (as unit of time) was introduced. The system was revised in 1873 and base unit of measuring length was changed to centimetre. This is known as **CGS** (centimeter-gram-second) system and remained in use for almost a century. However, even this system did not solve all problems. In 1954 the units were redefined by Conference Generale des Poids et Measures and in 1960 the final version of now internationally accepted system of measurements was published. This system is called *System Internationale* Units (International System of Units) or simply as SI.

The international system of units has been developed and agreed internationally. It has following important advantages:

1. It overcomes language barriers.
2. Enables an exchange of health information within a country and between nations to be made without misunderstandings, which arise when each country, or even a hospital within a country, uses its own units of measurements for reporting tests.
3. The international system (System Internationale, SI) of units is based on the **metre-kilogram-second** system and replaces both the **foot-pound-second** (IMPERIAL) system and the **centimeter-gram-second** (CGS) system.

STRUCTURE OF SI

SI comprises three types of units: base units, derived units and supplementary units. **Base units** are seven in number. Their symbols, quantity and values are well defined. These are shown in Table 2.1. **Derived units** are obtained by mathematical manipulation of one or more of the base units. Best example is the unit of volume. This unit is called cubic metre and is derived simply by cubing the base unit metre. It is written as m^3. An example of unit derived from two base units, metre and second is unit of speed. This unit is called **metre per second** and is written as m/s or m.s. A derived unit may involve more than two base units. For example, the unit of force is defined as that force which gives to a mass of 1 unit (1 kg) an acceleration of one unit ($1m/s^2$). As it is difficult to write such a lengthy unit so such units are given special names. Most of the names are those of scientists who made an outstanding contribution to the study of the field concerned. Thus the derived unit of force is given the name of **Newton** and is symbolised with N. While writing derived units certain principles must be followed. A horizontal bar, a stroke or a negative exponent, can denote a division. For example, speed can be written as m/s, or $m.s^{1}$. The last one is to be preferred. A multiplication, similarly, can be written with a dot on line, dot above the line or a space between the two. When writing complex symbols like mg/kg/day, great care should be taken.

Table 2.1: Base Units

Quantity	Units	Symbols
Length	Metre	m
Mass	Kilogram	kg
Time	Seconds	s
Electric current	Ampere	A
Thermodynamic Temperature	Kelvin	K
Luminous intensity	Candela	cd
Amount of substance	Mole	mol(M)

Table 2.2: Derived Units

Quantity	Units	Symbol	Derivation
Pressure	Pascal	Pa	N/m^3
Power	Watt	W	J/s
Electric Potential	Volt	V	W/A
Celsius temperature	Degree Celsius	°C	K
Absorbed dose radiation	Gray	Gy	J/kg
Activity, radiation	Becquerel	Bq	S^{-1}

It must be remembered that not more than one stroke should be used in the symbol for a unit unless ambiguity is removed with use of parenthesis. In the unit mg/kg/day, if written as such reader may appreciate it as (.mg/kg/day, or mg/(kg/day) while in fact it is the first one that is correct. Therefore, it is better to write (mg/kg)/day. Some derived SI units of medical interest are shown in Table 2.2. Supplementary

SI units are the units about which it is still not decided that whether these shall be placed in base unit or derived unit categories. These are not of concern to medical profession.

Table 2.3: Prefixes

Factor	Prefix	Symbol	Factor	Prefix	symbol
10^{18}	Exa	E	10^{-1}	Deci	d
10^{15}	Peta	P	10^{-2}	Centi	c
10^{12}	Tera	T	10^{-3}	Milli	m
10^{9}	Giga	G	10^{-6}	Micro	µ
10^{6}	Mega	M	10^{-6}	Nano	n
10^{3}	Kilo	K	10^{-12}	Pico	p
10^{2}	Hecto	H	10^{-15}	Femto	f
10^{1}	Deca	D	10^{-18}	Atto	a

Sometimes an SI unit is so large that it is inconvenient to write it. To overcome this problem SI has incorporated 16 prefixes, which can be written instead. These are given in Table 2.3. When a prefix is used, it is joined directly to the symbol or name of unit. For example red blood cell volume is stated in litre. By measurement it is 10^{-15} litres but by using symbol for both it is written fl (femtolitres). There are certain units, which are so commonly used that SI has allowed their use without changing them. These are shown in Table 2.4.

Table 2.4: Unchanged Units

Quantity	Unit	Symbol	Value in SI Units
Time	Minute	Min	60s
	Hour	h	3600s
	Day	d	86400s
Plane angle	Degree	°	H/180 rad
	Minute	‘	H/10800 rad
	Second	“	H/648000 rad
Volume	Litre	l	$1dm^3$
Mass	Tonne	t	1000kg

Another group of commonly used units has temporarily been retained. These are shown in Table 2.5.

Table 2.5 :Tmporarily retained Units

Unit	Symbo	Value in SI Units
Angstrom	A	10^{-10}m
Barn	B	$10^{28}m^2$
Bar	Bar	100,000Pa
Normal atmosphere	Atm	101325 Pa
Curie	Ci	3.7 X 10^{10} Bq
Roentgen	R	2.58 X 10^{-4} C/kg
Rad	rad, rd	10^{-2} Gy

Conversion of some conventional units into SI is given in Table 2.6. Symbols for units are always written in normal type whatever the format of text is and do not change into plural. For example Kilograms is written as kg and not as Kgs. Full stop is not used after the symbol unless the symbol is at the end of a sentence. Decimal shall be indicated with a coma or dot on the line. A raised dot, in SI, indicates sign of multiplication. For example **three decimal five** shall be written as **3.5** or **3,5**. If it is written as 3˙5 in SI it means 3x5 or 3*5.

Table 2.6: Conversion factors from conventional to SI and from SI to conventional units

Analyte	Old Unit	New Unit	To SI	From SI
Haemoglobin	g/100 ml	**g/L**	10	0.1
Red blood cell count	$10^6/mm^3$	**10^{12}/L**	1	1
White blood cell count	mm^3	**10^9/L**	0.001	1000
Platelet count	mm^3	**10^9/L**	0.001	1000
Haematocrit	%	**L/L**	0.01	100
MCV	μ^3	**fl**	1	1
MCH	Pg	**fmol**	0.06206	6.11
MCHC	g/dl	**mmol/L**	0.6206	1,611
Albumin	9/dl	**g/L**	10	0.1
Aldosterone (24h Urine)	mg	**nmol**	2.774	0.3604
Ammonium	mg/dl	**µmol/L**	0.5872	1.703
Ascorbate	mg/d)	**µmol/L**	56.78	0.01761
BUN	mg/dl	**mmol/L**	0.357 0	2.801
Base excess	meq/l	**mmol/l**	1	1
Bicarbonate.	meq/l	**mmol/L**	1	1
Bilirubin	mg/dl	**µmol/L**	17.10	0.05847
Calcium	mg/dl	**µmol/L**	0.249 5	4.008
Carbondioxide	mmHg	**Kpa**	0,133 3	7,502
Carboxy haemoglobin	%	**mol/mol**	0,01	100
Ceruloplasmin	mg/dl	**mg/L**	10	0.1
Cholesterol	mg/dl	**mmol/L**	0.025 86	38.67
Chloride	meq/l	**mmol/L**	1	1
Coproporphyrin	µg/dl	**nmol/L**	15.27	0.065 47
Corticosteroids	µg/dl	**µmol/L**	0.02759	36.25
Corticotrophin (ACTH)	Pg/ml	**pmol/L**	0.2202	4.541
Cortisone	µg/dl	**µmol/L**	0.027 74	36.04
Creatine	mg/d)	**µmol/L**	76.28	0.01311
Creatinine	mg/dl	**µmol/L**	88. 40	0.01131
Copper	µg/dl	**µmol/L**	0.1574	6.355
Cyanocobalamine	ng/dl	**pmol/L**	7.378	0.1355
Fibrinogen	mg/dl	**g/L**	0.01	100
Folate	µg/dl	**nmol/L**	22.60	0.044 14
Globulins	mg/dl	**g/L**	0.01	100
Glucose	mg/dl	**mmol/L**	0.0555l	18.02
Haptoglobin	mg/dl	**g/L**	0.01	100
Haemoglobin	g/dl	**mmol/L**	0.6206	1.611
Insulin	µU/ml	**pmol/L**	7.175	0.1394
Iron	µg/dl	**µmol/L**	0.1791	5.585
17-ketosteroids	mg	**Mmol**	3.467	0.2884
Lactate	mg/dl	**mmol/L**	0.1110	9.008
Lithium	mg/dl	**mmol/L**	1.441	0.684 1
Lipid total	mg/dl	**g/L**	0.01	100
Lipoprotein	mg/dl	**g/L**	0.01	100
Methaemoglobin	g/dl	**µmol/L**	620.6	0.001611
Magnesium	mg/dl	**mmol/L**	0.411 4	2.431
Myoglobin	mg/dl	**mg/dl**	0.5848	1.710
Oxygen	mmHg	**KPa**	0.1333	7.502
Oxygen saturation	%	**mol/mol**	0.01	100
Phosphates	mg/dl	**mmol/L**	0.3229	3.097
Phospholipid	g/1	**mmol/L**	1.292	0.774
Potassium	meq/l	**mmol/L**	1	1
Porphobilinogen	mg	**mmol**	4.420	0.2262
Protein	g/dl	**g/L**	10	0.1
Protoporphyrin	µg/dl	**µmol/L**	0.017 77	56.27
Sodium	meq/l	**mmol/L**	1	1
Transferrin	mg/dl	**g/L**	0.01	100
Triglycerides	mg/dl	**mmol/L**	0.01129	88.54
Thyroxin	µg/dl	**nmol/L**	12.87	0.07769
Triiodothyronine	ng/dl	**nmol/L**	0.01536	65.10
Urates	mg/dl	**µmol/L**	59.48	0.01681
Urea	mg/dl	**mmol/L**	0.1665	6.006
Urobilinogen	mg	**µmol**	1.687,	0.592 7
Uroporphyrin	µg	**nmol**	1.204	0.8308
VMA	mg	**µmol**	5.046	0.1982
Zinc	µg/dl	**µmol/L**	0.1530	6.538

STANDARDISED REPORTING OF LABORATORY RESULTS

Unification of measuring units is not the only requirement for producing laboratory reports,

which can be understood by every body in any part of the world. It also involves the use of recognised symbols, abbreviations and an internationally accepted format of report. Such symbols and abbreviations for some common parameters and quantities are given in Table 2.7. A result is reported in the following format:

Table 2.7: Symbols and Abbreviations

System/Quantity	Symbol/abbreviation
Arterial	Prefix a
Blood	B
Day	Prefix d
Erythrocyte(s)	Erc(s)
Fasting	Prefix f
Leukocyte(s)	Lkc(s)
Plasma	P
Patient	Pt
Serum	S
Spinal fluid	Sf
Urine	U
Volume	Vol
Molality	molal
Relative	rel
Difference	diff

1. Name of the system or its abbreviation
2. A dash or two hyphens
3. Name of the component beginning with capital letter
4. A comma
5. The quantity name or its abbreviation
6. An equals sign
7. The numerical value and the unit

UNITS IN CLINICAL ENZYMOLOGY

An international unit of enzyme activity is that amount of enzyme, which under defined assay conditions will catalyse the conversion of 1 µmol of substrate/min (see also ROLE OF ENZYMES IN CLINICAL LABORATORY on page 349). Results are expressed in international units/litre. In accordance with this definition the assay conditions for enzyme analysis must be specified. International units used in clinical enzymology are not the part of SI. A unit in enzymology is actually the activity of the enzyme required to convert substrate into a unit of product, which is measured. Since all methods of enzyme assay are dependent upon substrate, technique and temperature employed so the standardisation is difficult. For common enzyme assays international units have been described. These are always in units of activity per litre and are written as IU/L (Table 2.8).

Table 2.8: Conversion factors for Units in Enzymology

Enzyme	Procedure	Conversion factor
Acid phosphatase	King-Armstrong	1.7826
Alkaline phosphatase	King-Armstrong	7
α-Amylase	Somogyi	1.875
ALT (SGPT)	Reitman-Frankel	1
AST (SGOT)	Reitman-Frankel	1

3. BASIC LABORATORY EQUIPMENT

THE MICROSCOPE

The light microscope is one of the most basic and essential equipment used in any laboratory. It is used for visualising very small objects like cells, bacteria, parasites, their ova/cysts and crystals etc., that are otherwise not visible to the naked eye. It comprises a series of lenses, which magnify an illuminated small object several times to make it recognisable with the naked eye and to study its details. Such a microscope is called **compound** light microscope. Adjustment of the microscope's illumination and optical system for optimum contrast and resolution is crucial for accurate recognition of the image produced and studying its details. The capabilities of a light microscope can only be best utilised if the laboratory technologist or pathologist fully understands the basic principals of image formation, microscope components and their functions. Whether a light microscope is monocular (having one eyepiece) or binocular (having two eyepieces) or multi-head (used by more than one observers simultaneously) the basic components remain the same. It has three basic components:

- Foot piece
- Body
- Eye piece

Foot piece

It forms the base of the microscope and provides stability to the body and eyepieces. The light source, with or without its controls, is usually incorporated in the base. In some old or field microscopes a mirror is provided in place of a light source. This allows the use of natural or external source of light to illuminate the object. One side of the mirror is concave and is used when more intense light is required to illuminate a small field. The other side of the mirror is convex and is used when less intense (diffuse) light is required to illuminate a broad field.

Body

The body of the microscope is mounted on the foot piece. It holds a sub-stage **condenser**, a stage and a nosepiece. Sub-stage condenser is composed of a system of lenses and diaphragm. The intensity of light and the size of field illuminated by it are controlled by moving the condenser up or down and adjusting the aperture of the **diaphragm**. The stage is a device for holding the objects for examination. It has a hole in the middle over which the object is placed. Exactly underneath the hole is the sub-stage condenser. The stage may be a fixed stage with clips to hold the object in place. But in most microscopes it is provided with a mechanical device to move the object in both planes (**mechanical stage**). The device is marked on both axes for noting the grid reference of the field examined. This helps in localising the field in future examination of the same object. **Nosepiece** is the part of the body, which holds the objectives. In modern microscopes it comprises a revolving device to hold 4-5 objectives of different magnification. The device helps in bringing the required objective over the object for examination. An **objective** comprises a system of lenses, which magnify the image several times. Each objective is marked with a coloured line, which indicates its magnification. The magnification is also engraved on the objective in numeric along with other information. For example a dry high power objective has a blue line and is engraved with following:

Plan 40/0.65
160/0.17

This means that this particular objective has a magnification of x40 and has a numerical aperture 0.65 at a tube length of 160 mm when a cover glass of 0.17 mm thickness is used. Word **Plan** denotes the type of objective.

Following are the common objectives installed in an ordinary light microscope:

- Scanner: Red line, x4 magnification
- Low power: Yellow line, x10 magnification
- Dry high power: Blue line, x 40 magnification
- Oil immersion: White line, x100 magnification

Eyepiece

The observer, to look at the object under examination uses this part of the microscope. The microscope having one eyepiece is called **monocular** whereas the microscope with two eyepieces is called **binocular** microscope. The eyepiece consists of a system of lenses that further magnify the image produced by the objective. The magnification power of the eyepiece is inscribed on it e.g., x10. In binocular

microscope two eyepieces are installed in a tube provided with a prism to divert the incident light to both eyepieces equally. The observer can adjust the distance between two eyepieces (inter-pupillary distance) to his convenience. Movement of the eyepiece in the holding tube allows diopter setting for an individual observer.

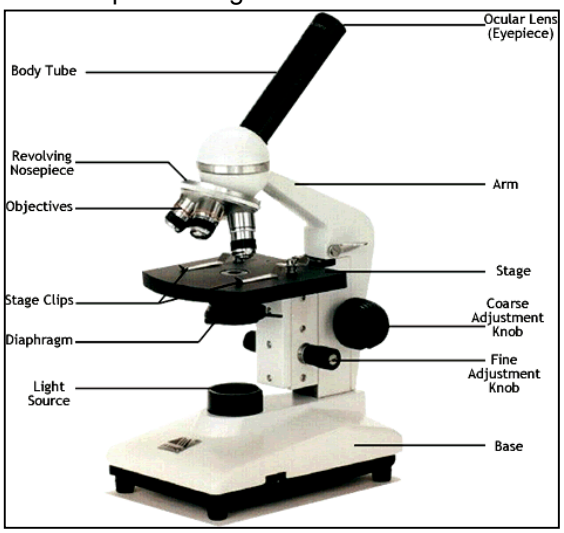


Figure 3.1: Simple Microscope

ESSENTIALS OF IMAGE FORMATION IN LIGHT MICROSCOPY

The light constitutes the raw material of the light microscopy. The light is a form of energy that travels in waves. **Wavelength** is the distance between two corresponding points on adjacent waves and determines the colour of light. The visible light is a mixture of seven different colours with wavelength (λ) in the range of 400-750 nm (Figure 3.3). The **frequency** (f), i.e. the number of variations per second, of these waves is responsible for differences in colour. Whereas the **amplitude**, i.e., vertical displacement of the wave from the optical axis determines the intensity or brightness. The light rays when pass from air to a dense medium e.g., the lens of the microscope, change their direction and speed. This is called **refraction**. The refractive index of air is 1.0 whereas that of glass and cedar wood oil is 1.5. If the refractive index of all the media is same it results in better magnification. Similarly light rays, while passing through an object, loose some of their intensity. This is called **absorption**. Not all the light rays succeed in entering from one medium to other. Some of these change their direction. This is called **diffraction**.

Lenses

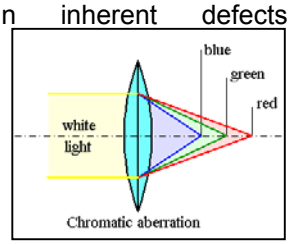
A lens is an optical element composed of glass or other transparent material. There are two basic types of lenses. First are positive, convex lenses, which cause light rays passing through them to converge to form an image. Second are negative, concave lenses, which cause light rays passing through them to diverge to form an image. Each type of lens has specific ability to delineate details of an object under examination. This is called **resolution**. It is the smallest distance (in μm) between two structural elements that can still be visually distinguished from each other. The resolution (R) of the lens is determined by its **numerical aperture** (NA) and the wavelength (λ) of the illuminating light. Shorter the wavelength better is the resolution thus:

$$R\,(\mu m) = \frac{1.2\lambda\,(\mu m)}{2NA}$$

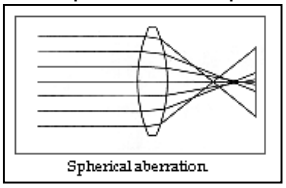
The numerical aperture (NA) is the ratio of the diameter of the lens to its focal length. It can be calculated by the formula:

NA = N Sin U

Where N is the refractive index and U is the angle of aperture. **Focal length** is the distance between the lens and the object from which all rays of light are brought to a point or focus. All lenses have certain inherent defects (aberrations). These are of six types but two are important. **Chromatic** aberrations are responsible for colour fringes on the margins of image.



Spherical aberrations are responsible for poor image definition and contrast. Spherical aberrations create curved images of flat objects. These are corrected by using a combination of lenses of various shapes and types in an objective.



Working distance is the depth of space in mm between the top surface of the object and the front surface of the objective. It reduces with increase in power of the lens. For this reason high power lenses are provided with spring loaded front part to avoid damage to the lens or object.

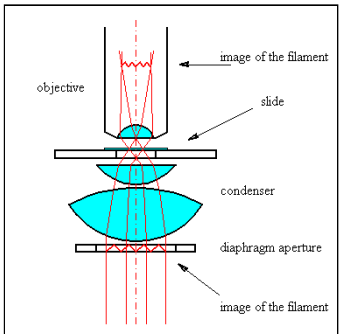
Depth of focus is the distance through which all parts of the object image are clearly in focus simultaneously.

Field of view is the area of an object that can be seen.

Magnification is the degree of enlargement of the visual image of an object produced by the optical system of the microscope. There are two magnifying optical systems in the microscope, objective and the eyepiece. Final magnification of the image is the product of magnification of **objective** and the **eyepiece**. For example when using an objective of x40 and an eyepiece of x10 magnification, the final magnification of the object will be 40x10=x400. Increasing magnification reduces the depth of focus as well as field of view. Also with increasing magnification greater amount of light is required to illuminate the field.

HOW TO OPERATE A COMPOUND LIGHT MICROSCOPE

1. The microscope should be placed on a level bench, which should be free of vibrations.
2. The power socket, to which the microscope is plugged, should not be loose and sparking.
3. The height of the microscope or chair should be adjusted in such a way that the eyes of the user are right on the eyepieces while maintaining the normal curvatures of the backbone.
4. The microscope should then be adjusted for the optimum resolution and contrast to ensure maximum definition of specimen details. It can be done by using **Köehler technique** as under:



Condenser and Köhler illumination

   - Turn on the microscope at very low illumination and give 1-2 min to the filament of the bulb to warm. Then adjust the light intensity.
   - Place the specimen on the stage, switch to x10 objective and focus.
   - Close the iris diaphragm of the sub-stage condenser and raise the sub-stage condenser to the top “stop”.
   - Close the field iris diaphragm of the light assembly in the body.
   - Move the sub-stage condenser down until the image of the field iris diaphragm is in sharp focus.
   - Now re-focus the specimen.
   - Centre field diaphragm image by using adjustment screws in the condenser.
   - Enlarge field diaphragm image until it is just out of the field of view and the entire area under observation is illuminated.
   - Remove one eyepiece and look down the tube.
   - Adjust the aperture of diaphragm while observing the circular beam of the light so the light beam fills 75% of the field.
   - Replace the eyepiece. Adjust the diopter setting and inter-pupillary distance.

Place your forearms flat on the surface of the table while using microscope. Periodically look away, preferably out of window or to a picture or any pleasant object.

OIL IMMERSION MICROSCOPY

Oil immersion microscopy is extensively used to identify very small objects and to study finer details of the cells. It requires the use of specially constructed objectives with a small working distance. Air (refractive index 1.0) in the light path of the object space is replaced with oil (refractive index 1.5-1.6). This improves resolution. Oil immersion objectives of various magnifications are available but the most commonly used has a magnification of x100. Following procedure should be adopted for oil immersion microscopy:

1. Adjust the microscope.
2. Place the object on the stage and focus with x10 objective.
3. Select the viewing area.
4. Rotate the objective out of light path.
5. Place a drop of oil over the object, in the centre of light beam.
6. Watching from the side, carefully swing in the oil immersion objective.
7. Focus carefully using fine adjustment knob.
8. After examination wipe off the oil and clean the objective as well as the object with a piece of soft tissue paper.

CARE OF THE MICROSCOPE

Microscope is very delicate equipment. Proper care not only enhances precision but also increases its life. Following points are helpful in the care of microscope:

1. Protect from heat.
2. Clean it daily. When not in use, keep it covered with a plastic cover or a piece of cloth but not with mesh gauze.
3. Clean the objectives with soft tissue paper soaked in xylol and then with lint free cloth. Be careful as excess of xylol may dissolve

the cement with which lens is fixed in the objective and may trickle into it. Do not clean with alcohol.
4. Remove the dust from the eyepieces with the help of soft tissue paper.
5. Always use soft tissue paper or lint free cloth for cleaning lenses and never rub but wipe gently. This protects lenses from scratches.
6. Switch off the power at the end of microscopy session.

TROUBLE SHOOTING AND REMEDIES

1. No light: This may happen if the power connection is loose, the bulb is loose or fused, brightness control dial is at lowest level, objective is not clicked in place, diaphragm is completely closed or not centred or fuse is blown. The cause should be recognised and removed.
2. Insufficient light: This may result from low set brightness control dial, too low condenser and closed condenser diaphragm. Check and correct accordingly.
3. Too bright light: Brightness control set too high for the objective being used.
4. Flickering: Flickering results from loose power connection, defective bulb socket, corrosion of bulb pins and improperly installed bulb.
5. Does not focus with high objective: The specimen slide is placed up side down.
6. Bubbles or dark waves across the field: Contact between the oil and oil immersion objective is broken. Clean the slide and add more oil.

SPECIAL TYPES OF MICROSCOPES

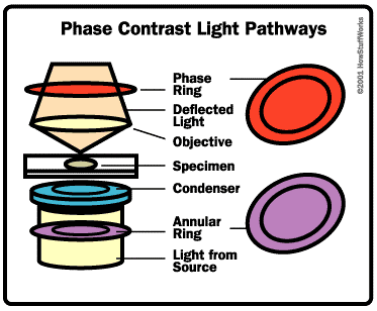
Dark ground microscope

It is also called dark field illumination microscope. There are certain microorganisms, which are very difficult to stain e.g., spirochetes. To visualise them under microscope dark field illumination is used. The microorganisms appear bright against a dark background. It is similar to dust particles seen in a beam of light from a ventilator in a dark room. In this microscope a special condenser with a central black area is placed just behind the objective. A dark ground, phase contrast microscope can be made from an ordinary microscope. For this, cut out a thick talc sheet of the size of a filter. Colour the central two third with black ink. Place it along the filter in the holder below the condenser.

Fluorescent microscope

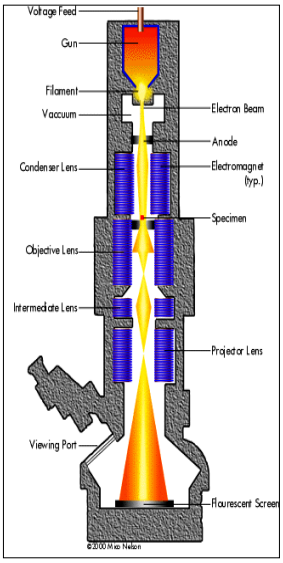
Certain dyes have the characteristic of glowing when exposed to ultraviolet light. In fluorescent microscope the object is stained with these (**fluorochrome**) dyes. The light source of microscope is replaced with a source that provides only ultraviolet light. The object appears as a glowing particle against a dark background. **Rhodamine** and **Auramine** are commonly used flourochrome dyes. If an antibody is attached to these flourochrome dyes, the presence of a specific antigen can be detected. This is called **immunofluorescent** microscopy (See also page 227).

Phase contrast microscope



This microscope is used for observing unstained living organism with good contrast and high resolution. It is useful for the study of structure of large microorganisms, tissues and cells. Unstained bacteria and cells consist of alternate strips of materiel of different refractive indices that cause the light to acquire small phase differences. These differences are exaggerated by causing the direct and diffracted rays to pass through different thickness of glass in phase plate. Direct and diffracted light beams are then recombined to produce an image.

Electron microscope



This microscope is used to see viruses or parts of cells smaller than the limits of resolution of the light microscope. It utilises a beam of electrons instead of visible light and electromagnetic fields in place of optical lenses. An object forms an image in the electron microscope because its solid content scatters the electron beam and so casts a shadow in the electron beam. The image cannot be seen with eye. Instead, it is focused on a screen and/or is photographed. Further magnification and resolution can be obtained by enlarging the photographs.

COLORIMETERS AND PHOTOMETERS

Ordinary white light (sun light) or near white light (tungsten or tungsten halogen filament light) is the visible part of a continues spectrum of electromagnetic energy waves. It is composed of a mixture of energy waves in a range of 400-700 nm (Figure 3.3).

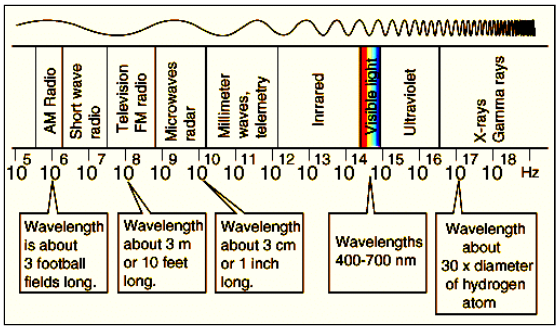


Figure 3.2: The Electromagnetic Spectrum

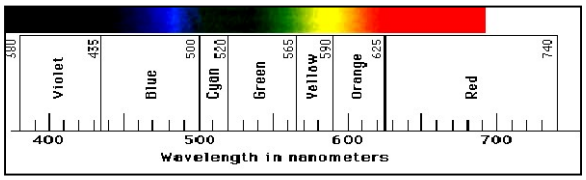
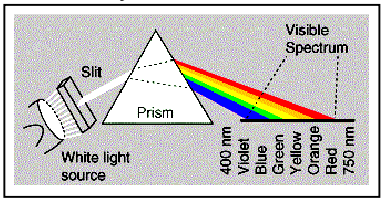


Figure 3.3: The visible spectrum and wavelengths

On both sides of this visible range the spectrum becomes invisible to naked eyes. Red colour has the shortest wavelength whereas violet colour has the longest. Below 400 nm is the ultraviolet range and beyond 700 nm is the infrared zone. The wavelengths (spectral colours) can be separated by a dispersive medium such as water droplets in air (rainbow) or a glass prism, more effectively by a **diffraction grating** in an instrument. The seven colours seen by this dispersion can be remembered by the word 'VIBGYOR' (Violet, Indigo, Blue, Green, Yellow, Orange, Red). A spectral colour is composed of a single wavelength. Most colours are composed of a range of wavelengths (Table 3.2) but a light of a single wavelength is called **monochromatic** light corresponding to a single colour. The intensity of the colour is proportional to the amount of waves of that particular wavelength absorbed. In practice the pure colours are defined in terms of wavelengths. Based on these principals, various instruments have been developed to quantitate coloured and un-coloured substances in clinical samples.



COMPLEMENTARY COLOURS

Complementary colours are the pair of opposite colours which when combined together in the ratio in which they are present in the visible spectrum give rise to white light and thus complement each other. These pairs are given in the Table 3.1. Conventionally it is believed that primary colours are seven. Here the eighth colour is magenta, which is a combination of red and violet.

COLORIMETRY

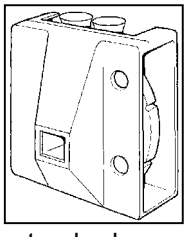
Measurement of colour intensity of a solution is known as colorimetry. When light of complementary colour is passed through a coloured fluid it absorbs certain amount of that light (wavelength) and transmits the rest (selective absorption). This process is responsible for the specific colour of that liquid. This forms the basis of estimation of various chemical substances in blood and body fluids. These substances are allowed to react with certain reagents to produce coloured compounds. The intensity of produced colour is compared with colour produced by a known amount (standard) of the same substance in similar reaction and the concentration is calculated provided:

1. The intensity of colour produced is proportional to the quantity of that substance (Beer-Lambart law).
2. No other interfering substance may be present, which can produce similar colour reaction.
3. The colour remains stable for long enough to allow its comparison or measurement.

Table 3.1: The complementary pairs of colours.

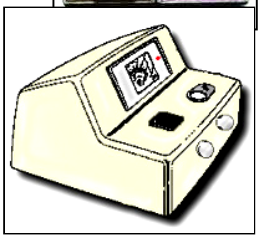
Colour	Complimentary colour
Violet	Yellow
Indigo	Orange
Blue	Red
Green	Magenta

COLORIMETERS



A colorimeter is an instrument, which measures the intensity of colour produced in a solution. These are of two types. One type of colorimeter compares the colour intensity simultaneously with standard called comparators. Comparing standard may be in the form of a disc (**Lovibond**) or a tube

(**Sahli's,** page 250) or it may have to be put in a separate tube but seen simultaneously (plunger colorimeter). The second type of colorimeter measures the intensity of colour of test and standard solutions separately and concentration is then calculated.



Photoelectric Colorimeter

In this instrument light of a known wavelength (complimentary colour) is passed through the coloured solution and amount of light absorbed (A) or transmitted (%T) is measured with the help of a photocell. The wavelength is selected by use of different coloured filters. There are five essential components of this instrument (Figure 3.4):

1. **Light source**: Lamps convert electrical energy into radiation. Different designs and materials are needed to produce light in different parts of the electromagnetic spectrum. An ordinary tungsten filament bulb, tungsten halogen or a quartz lamp emit a continuous spectrum of light.
2. **Filter**: Filters separate different parts of the electromagnetic spectrum by absorbing or reflecting certain wavelengths and transmitting others (Table 3.2). These are of two types:
   a. *Colour filters* are glass substances containing absorbing species. These are made up of a layer of coloured material (gelatin) pressed between two layers of thin glass that absorbs light of certain wavelengths. A typical example is a cut-on colour filter, which blocks short wavelength light such as an excitation source, and transmits longer wavelength light such as fluorescence that reaches a detector.
   b. *Interference filters* are made of multiple dielectric thin films of a substance. They use interference to selectively transmit or reflect a certain range of wavelengths.

Filter allows a narrow band of light of a selected wavelength (colour) to pass within a narrow range of wavelength and absorbs the rest. It is important that they are kept dust free and examined periodically for scratches, cracks and fading of colour because, these defects will affect their sensitivity.

Table 3.2: Wavelengths of Colour Filters

Colour	Wavelength (nm)	Colour	Wavelength (nm)
Red	680-700	Green	500-520
Orange	600	Blue	460-480
Yellow	580	Violet	410-430

3. **Sample cuvettes**: These are tubes or cups of standard bore and wall thickness, made up of colourless, high quality glass. For measurements in UV range glass cuvettes are unsuitable as they themselves absorb UV light. For such measurements special quality cuvettes made of quartz glass are needed. Other tubes should not be used.
4. **Photocell or Photomultiplier tube (PMT)**: It converts the transmitted light falling on to it into electric current, the amplitude of which is proportional to the amount of light transmitted. It is a very sensitive device and deteriorates with use.
5. **Galvanometer**: It measures the amount of current produced by the photocell. It is calibrated according to the colour intensity. Usually have two scales, one for absorbance (A) and other for transmission (%T). The output of photocell can also be directed to a digital display.

Operation:

1. Select or insert an appropriate filter and then switch on the equipment and allow time to warm up.
2. Insert the tube containing blank in the cuvette holder. Adjust galvanometer to read zero absorbance (or 100% transmission) with adjustment knob.
3. Replace blank with the test solution. Allow the needle or digital display to become stable and then note the reading.
4. Repeat the process with the tube containing standard and note the reading.

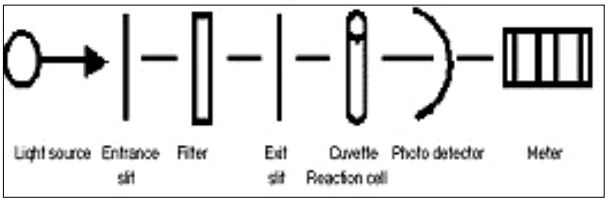


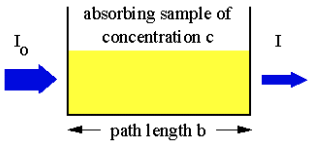
Figure 3.4: Essential parts in the light path of a photoelectric colorimeter

Modern absorption instruments can usually display the data as transmittance, %-transmittance, or absorbance. Measuring the amount of light that a sample absorbs and applying Beer's law can determine the unknown concentration of an analyte. If the molar absorptivity coefficient (a) is not known, the unknown concentration can be determined using a working curve of absorbance (standard curve)

versus concentration derived from a series of standards (Figure 5.9).

Calculation:
Transmittance (T) is defined as:

$T = I / I_o$



where I is the light intensity after it passes through the sample and I_o is the initial light intensity. The relationship between absorption (A) and transmittance or transmission (T) is:

$A = -\log T = -\log (I / I_o)$
According to the Beer-Lambert law (or simply, Beer's law) the linear relationship between absorbance and concentration of an absorbing species is given by the formula:

Absorbance (A) = a*b*c
Where;
a = molar absorptivity constant
b = path length
c = concentration

OR the two equations for unknown (U) and standard (S) can be written as
$A^U = a*b*c^U$ (1) and
$A^S = a*b*c^S$ (2)
By removing path length (**b**) and molar absorptivity constant (**a**) from both equations and combining them together, the equation becomes:

$c^U/c^S = A^U/A^S$ OR $c^U = A^U/A^S \times c^S$

Therefore, concentration of unknown is equal to the ratio of colour intensities of unknown and standard multiplied by the concentration of standard. Thus the final equation can be represented by

$$\text{Concentration}^{U} = \frac{\text{Absorbance}^{U}}{\text{Absorbance}^{S}} \times \text{concentration}^{S}$$

Limitations of the Beer-Lambert law
The linearity of the Beer-Lambert law is limited by chemical and instrumental factors. Some of these include:

1. Deviations in absorptivity coefficients at high concentrations
2. Scattering of light due to particles in the sample
3. Fluorescence or phosphorescence of the sample
4. Changes in refractive index at high analyte concentration
5. Non-monochromatic radiation, deviations can be minimised by using a relatively flat part of the absorption spectrum such as the maximum of an absorption band
6. Stray light

Care

1. Do not switch on without a dark filter in place. Direct light will damage the photocell.
2. Protect filters from scratches, dust and direct prolonged light. When not in use, spare filters are kept in their packing.
3. For taking a reading, needle must be allowed to settle down.
4. Keep the glass tubes clean.
5. Keep the equipment covered, when not in use, to protect from dust.

SPECTROPHOTOMETERS

These are advanced instruments utilising the principles described in photoelectric colorimeter. The main difference is that the light of required wavelength is obtained by a prism or diffraction grating incorporated in a monochromator. Wavelength is selected electronically. The reaction mixture is placed in a cuvette of colourless glass and of known internal volume and wall thickness. The light is first passed through a monochromator and then through the sample tube containing reaction mixture. The transmitted light then falls on a photocell or photomultiplier tube, which converts it to electrical energy. This in turn, is measured by a galvanometer and displayed. A number of spectrophotometers are available, the most popular being the **spectronic** series. Some advanced models are modified to work at ultraviolet wavelengths. This not only increases the range of tests but also permits the use of micro-methods. Deuterium lamps are the UV source in UV-VIS absorption spectrophotometers. Mercury and xenon arc lamps are used to excite fluorescence. Some spectrophotometers measure change in absorbance per unit time (Δ_A) during incubation and then calculate the concentration based on rate of reaction (kinetic measurements). These instruments can be programmed to give concentration directly. As there are a number of models, therefore, use, care and troubleshooting are to be followed according to the instructions of the manufacturer.

FLAME PHOTOMETER

Flame photometer is an instrument used for quantitation of certain metals such as sodium (Na), potassium (K) and lithium (Li).

Principle
When a metal is heated in hot part of a flame, it

absorbs thermal energy that transforms it into radicals and atoms. Further heating shifts its electrons into outer most, high-energy orbits. When these are cooled in cooler part of the flame, the absorbed thermal energy is emitted as light energy. Each element produces light of a specific colour (wavelength) (Table 3.3) and the intensity of that colour is proportional to the quantity. Selecting the appropriate wavelength for that element and measuring the change in intensity of light emitted by the flame quantitates the analyte. This principle is employed in **emission flame photometer**. As only 1-5% atoms of a substance are excited, this type of flame photometer is not sensitive enough for quantitation of trace elements. If the light of a particular colour (wavelength) is passed through the flame, the un-excited atoms of the element in the flame will absorb it. The decrease in the intensity of light is then measured from which the element is quantified. This is the principle of **atomic absorption flame photometer** (see Figure 3.5). As this instrument can measure up to 95-99% un-excited atoms, it is more appropriate and sensitive for quantitation of trace elements.

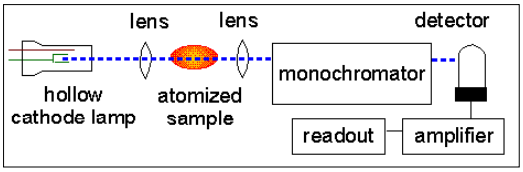
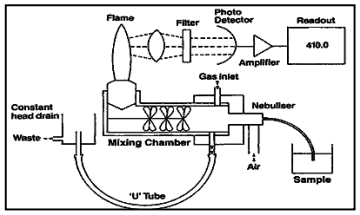


Figure 3.5: Schematic diagram of atomic absorption spectrophotometer

Components of emission flame photometer

1. **Nebulizer**: This is the most important part of a flame photometer. It provides a steady, fine spray of uniform sized droplets of test solution. It acts by directing a jet of air or oxygen under pressure across the end of a capillary, the other end of which is dipped in the solution. The solution is sucked into the capillary by Venturi effect.



2. **Burner**: Specially designed gas burner with a series of holes is used. When the gas burns in the presence of air or oxygen a series of clear blue cones are produced without yellow streaks. The temperature achieved is usually in the range of 2000°C when air and gas mixture is burnt. In case of oxygen and gas mixture the temperature in the range of 3000°C may be achieved. Commonly used gas fuel is propane or natural (Sui) gas.
3. **Wavelength selector**: It may be an appropriate filter or a monochromator.
4. **Reflector**: It collects the emitted light and reflects it on to a photo-detector.
5. **Photo-detector**: Converts light energy into electrical energy.
6. **Output device**: It may be a galvanometer or a digital display consisting of LEDs.

Table 3.3: Wavelength for commonly measured elements

Element	Wavelength (nm)	Colour
Sodium	589	Yellow
Potassium	766	Deep red
Lithium	671	Red
Calcium	554	Yellow green

Operation

1. Prepare appropriate dilution of the test specimen and standard solution. Since most commonly used test specimen is serum or plasma, higher dilutions are required to reduce the viscosity due to proteins. Viscous solutions cannot be nebulized adequately. The dilution also depends upon the expected concentration of the substance to be quantified.
2. Switch on the electric supply to the equipment.
3. Switch on the compressor to provide air or oxygen.
4. Open the gas valve and ignite the flame.
5. Adjust air and gas mixture to yield clear blue cones of flame.
6. Dip the outer end of nebulizer capillary in a container of de-ionised water and adjust the reading to zero.
7. Insert an appropriate filter or select the required wavelength and again adjust the display to zero with de-ionised water.
8. Replace container of de-ionised water with a container of standard solution and adjust the reading when stabilised to the concentration of the standard (calibration).
9. Reset zero with de-ionised water.
10. Replace this container with container of test solution and note the reading after it is stabilised.
11. Run de-ionised water again to clean the nebulizer.
12. Close the gas supply.
13. Switch off the compressor.

14. Switch off the electric supply.
15. Read the result from standard curve or calculate it using the same formula as in spectrophotometry.

Precautions

1. Only de-ionised water is to be used in preparing dilutions of test and standard solutions.
2. Gas regulator knob should be near minimum before opening the main gas supply to avoid explosion.
3. Precautions for use of spectrophotometer should also be followed.

Maintenance

1. Gas supply (Sui gas/cylinder) should be checked daily for any leakage and quantity of gas left in the cylinder.
2. Burner should be cleaned periodically to remove the deposited salts and proteins.
3. Nebulizer needs to be cleaned periodically.
4. Change the capillary tube when clogged or decolourised.
5. Compressor needs to be checked periodically for proper functioning.

WATER BATH

Water bath is an instrument used for maintaining a uniform temperature throughout the fluid contained in a glass container by keeping it in pre–heated water. It also prevents excessive evaporation of the fluid being heated. It allows the heating of small amounts of fluids over a period of time without changing the concentration of constituents by evaporation. It is also used when several tubes are to be handled while maintaining the temperature of the contents, e.g., in coagulation tests.

Components

1. A trough of insulated metal, usually stainless steel or of heat resistant glass with or without an insulated lid.
2. An electric element to heat the water contained in the trough.
3. A propeller or stirrer to circulate the water in the trough in order to maintain a uniform temperature throughout the trough.
4. A thermometer to check the temperature. This may be in-built or placed separately in the trough.
5. A thermostat to maintain the temperature at a constant level.

Operation

1. Fill the trough with clean (preferably distilled or deionised) water to a desired level and then switch it on.
2. Set the thermostat to desired temperature and allow the water to warm to that temperature. Check the temperature from the thermometer.
3. Place the containers to be warmed or incubated in the trough.
4. For prolonged incubation plug the containers with cotton wool to prevent tickling of water of condensation into them. Close the lid of water bath.

Precautions and maintenance

1. Clean the interior of trough and change the water daily or use deionised water. This will prevent encrustation of trough, stirrer, heat probe and thermostat with salts contained in raw water. It will also prevent the growth of fungi and algae.
2. Keep the lid closed when not in use to prevent evaporation of water.
3. Periodically check and counter-check the water temperature with internal as well as with an external thermometer. The thermometer should be placed in such a way that it is away from the heating element and the walls.

LABORATORY CENTRIFUGE

A centrifuge is a device that accelerates gravitational separation of substances that differ significantly in their masses.

Components

Centrifuges contain following components:

1. A rotor or centrifuge head
2. A drive shaft
3. Motor
4. Hanging buckets
5. Power switch
6. Timer
7. Speed/gravity control
8. Tachometer
9. Brake
10. Protective shield to minimise aerosol
11. Safety lock

Uses

1. It separates particulate materials from a solution in which they are suspended. For example:
   a. Removing cellular elements from blood to provide cell free plasma or serum for analysis.
   b. Concentration of cellular elements and other components of biological fluids for

microscopic examination or chemical analysis.
   c. Elimination of chemically precipitated proteins from an analytical specimen.
   d. Separating protein bound or antibody bound legend from free legend in immunochemical or other assays.
2. Separate two liquid phases of different densities.
   a. Extracting solutes in biological fluids from aqueous to organic solvents.
   b. Separating lipid components, e.g., chylomicrons from other components of plasma or serum and lipoproteins.

Types of centrifuge

Centrifuges generally may be classified into following types:

1. **Horizontal head or swinging buckets type**: It allows the tubes, placed in the cups of the rotor, to assume a horizontal plane when the rotor is in motion and a vertical position when it is at rest. During centrifugation, particles travel in a constant manner along the tube while the tube is at right angles to the shaft of the centrifuge. Thus the sediment is distributed uniformly against the bottom of the tube. The surface of the sediment is flat. Supernatant liquid is simply removed by a pipette with negligible disturbance of the packed sediment. It is ideal for separation of erythrocytes from plasma or of a protein precipitate from a solution.
2. **Fixed angle or angle head**: Tubes are held in a fixed position at angles from 25-40° to the vertical axis of rotation. Particles are driven outward horizontally but strike the side of the tube so that the sediment packs against the side and bottom of the tube with the surface of the sediment paralleled to the shaft of the centrifuge. As the rotor slows down and stops, gravity causes the sediment to slide down the tube and usually a poorly packed pellet is formed. It allows more rapid sedimentation of small particles as the fixed angle rotors can be run at a higher speed.
3. **Axial type**: It is a centrifugal concept that allows tubes of blood to be spun in a vertical orientation.
4. **Ultracentrifuge**: They are very high-speed centrifuges that usually use fixed head rotors. Mostly used in the separation of lipoproteins and ultra-microscopic particles. As considerable heat is generated during their operation, as a result of friction, so they are always provided with a refrigerated chamber.
5. **Special types**: There are some special types of centrifuges for specific purposes. Mechanically they fall under one of the above-mentioned types. The three most important types are:
   a. Immunofuge or Serofuge: This type of centrifuge is used in immuno-haematology. It is a horizontal head centrifuge with a fixed tube size head and fixed speed. It is commonly used in blood bank for spinning down the red blood cells.
   b. Cytospin: This is a horizontal head centrifuge having fixed speed and time. It is provided with special devices in the swinging head, which allow the cells in fluid phase to settle down on a glass slide. Because of the slow speed morphology of the cells is not disturbed. It is used for cytology.
   c. Blood bag centrifuge: This is also a horizontal head centrifuge but is provided with large buckets to hold blood bags. This is used in preparation of blood components i.e. packed red cells, platelets and plasma etc.
   d. Gerber centrifuge: This is a special centrifuge. It can hold and spin the Gerber tube, a special glass tube for milk analysis.

Operation

1. Only those tubes that are recommended by the manufacturer of the centrifuge should be used. The tubes should have a tapered bottom, particularly if the supernatant is to be removed.
2. The rotor must be properly balanced. Specimen tubes should be placed on opposite pans of a balance and equalised in weight. The placement of the tubes should also be symmetrical. Tubes filled with water may also be used to equalise the weight. The total weight of each rack should not exceed the limit stated by the centrifuge manufacturer.

   Imbalance of the rotor causes vibration that may increase wear and tear in the centrifuge and more frequent breakage of the tubes.
3. The lid should then be closed and locked.
4. Required time for centrifugation should be adjusted with the timer knob.

5. The centrifuge should then be switched on and allowed to attain speed for required centrifugation force, which should be adjusted with speed/gravity knob.
6. Lid should not be opened until rotor has completely stopped.

Maintenance

1. Cleanliness of a centrifuge is important in minimising the possible spread of infectious agents such as hepatitis viruses or mycobacteria. In case of breakage the racks and the chamber of the centrifuge must be carefully cleaned. Any spillage should be considered a biohazard and dealt accordingly.
2. Broken glass embedded in cushions of the tube holders may be a continuing cause of breakage if cushions are not inspected and replaced in the cleanup procedure.
3. The speed of a centrifuge should be checked at least once every 3 months, by stroboscopic light or a vibrating read external tachometer of known accuracy.
4. The centrifuge timer should be checked weekly against a reference timer such as stopwatch and the difference should not be more than 10%.
5. The temperature of a refrigerated centrifuge should be measured monthly under reproducible conditions and should be within 2°C of the expected temperature.
6. Commutators and brushes should be checked at least every 3 months. They should be replaced when they show considerable wear.

BALANCE

Balance is a device used for weighing things. Two types of balances are used in the laboratory:

1. **MECHANICAL BALANCE**: These are subdivided into various types depending upon the number of pans, reading mechanism and precision.
   a. **Trip balance**: This type of balance consists of two pans of equal size suspended with a beam that is supported in the centre of gravity by the edge of a sharp fulcrum. Substance to be weighed is placed in the right-hand pan and is counter balanced with known weights placed in the left-hand pan. Aligning the position of beam arm-bridge indicates correct weight. It is not precise and is used for weighing heavy things.
   b. **Spring balance**: This is a single pan balance used to weigh heavier things. The pan is attached with a spring, which stretches with weight. The weight is indicated on a scale by a pointer attached to the spring. It is not precise.
   c. **Analytical Balance**: These can be of two-pan type or one-pan type. Two-pan type mechanically operates on the same principle as trip balance. However, its beam is provided with side screws for fine adjustment of beam to zero weight and a pointer in the centre, which moves on a scale. It is relatively more precise. Single pan type has a beam of unequal arms. One arm is provided with a pan to place article for weighing, whereas it is counterbalanced by a single weight located at the opposite end of the beam. It is most precise among mechanical balances.
2. **ELECTRICAL/ELECTRONIC BALANCE**: It is a single pan balance and employs magnetic field to counteract the weight placed on the pan. The pan is attached to a coil, which is placed in a magnetic field generated by electric current. When more weight is placed on the pan more current is required to produce the magnetic field. This increase in current flow is converted by a microprocessor into numerical value for weight, which is displayed on a panel. These are of two types. One hanging pan type, usually protected in a glass case for weighing very small amounts. The other is top loading type commonly used for weighing larger quantities.

Operation

1. Place the paper or plastic weighing boat in the centre of the pan.
2. Perform tarring by pressing Tar button on the panel.
3. Place the substance to be weighed on the weighing boat with the help of a scoop or spetulum. The amount should roughly be slightly more than the required weight.
4. Reduce the substance with spetulum gradually until desired weight is indicated on the display panel.

Calibration

Balance needs to be periodically checked for accuracy. This is done by weighing a 100 g (or an appropriate) standard weight. The variation

should be <0.5%. If not, the balance should be adjusted accordingly.

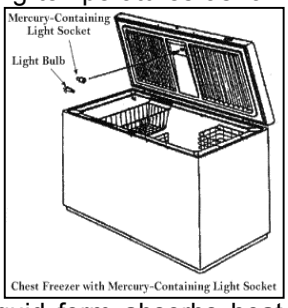
Precautions and maintenance

1. These should be protected from rusting and tripping mechanism & should be periodically cleaned.
2. Analytical balances should be kept in a glass box to keep these dust-free.
3. Balances must be placed on an absolute level surface for correct weighing. Analytical balances are usually provided with a spirit level and adjustable legs for levelling.
4. The surface on which the balance is placed should not be vibrating.
5. The pans should be absolutely clean.
6. Place the weighing object in the centre of the pan.
7. Always use standard weights. All weights should be placed in a dust-proof box. Small weights should be handled with forceps.
8. Material to be weighed should be placed in a pre-weighed plastic tray, boat or glazed paper that could be easily washed.
9. Close the door of the cabinet while weighing.
10. Do not weigh a substance when it is hot or cold.
11. Do not weigh a quantity that is beyond the permissible limits for the balance.
12. Keep the balance with door of the cabinet closed and switched off when not in use.
13. Use dust cover.
14. Clean the pan after each use.

COLD INCUBATORS, REFRIGERATORS, FREEZERS AND DEEP FREEZERS

All these equipment are used to provide temperatures well below room temperature (22-25°C), usually in the range of 2-8°C, although the temperature range varies according to the need of the laboratory as follows:

1. **Cold Incubator** operates between the temperature range 0-25°C as it is used for incubation or storage of fluids, blood and culture specimens etc. The control of temperature is more precise than refrigerator and some are provided with temperature recorder and alarm system. The blood bank is a special type of cold incubator, which operates in temperature range of 2-8°C.
2. **Refrigerator** commonly operates on temperature from 2-10°C. They are used to store laboratory reagents, body fluids, tissues etc. Some are provided with a freezer compartment.
3. **Freezers** operate at temperatures below 0°C and temperatures as low as -20°C can be reached in some. These are used to store sera, biological reagents and tissues etc., over a long period of time.
4. **Deep freezers** usually operate at temperatures below -20°C. Some are capable of maintaining temperatures as low as -80°C. They are used for storage of sera, body fluids, biological reagents, tissues etc., for periods extending over years.



Chest Freezer with Mercury-Containing Light Socket

Principle

A compressed gas in liquid form absorbs heat from the interior of the cabinet and expands into gaseous form. It is then taken back to the compressor to be compressed again into liquid form and in the process it gives away the heat absorbed to the exterior. Commonly used gas is Freon but for lower temperatures other gases like liquid nitrogen are also used.

Components

These consist of following components:

1. **Compressor**: It compresses the gas into liquid form.
2. **Condenser fan**: It provides cooling to the compressor.
3. **Condenser coils**: In these coils gas turns into liquid and gives away the heat.
4. **Evaporator fan**: It cools the evaporating system.
5. **Evaporator coils**: These absorb heat from inside the cabinet and vaporise the liquid gas.
6. **Thermostat**: It sets the temperature limit inside the cabinet.
7. **Defrost system**: Prevents frosting. This is only installed in cold incubators and refrigerators.
8. **Cabinet**: To accommodate articles for cooling. It is insulated and divided into compartments.
9. **Door switch and bulb**: Opening the door of the cabinet releases the switch that turns the bulb on. Closing the door presses the switch that turns the bulb off.
10. **Others**: Some are provided with temperature recorder to monitor the temperature inside the cabinet. Some are provided with alarm that goes on if the temperature crosses the set limits. Some are provided with digital display for the

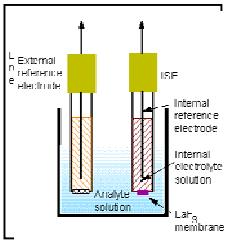
temperature inside the cabinet. Others have fan inside to circulate cold air to all areas.

Care and maintenance

1. The equipment should be placed against a fire resistant wall maintaining a distance of 6-8 inches to allow free circulation of air. It should be placed on a wooden stand. It must be in horizontal position. The level should be checked with the help of a spirit level.
2. A three-pin plug of appropriate amperage should be used to plug in the equipment. Ensure that socket is properly earthed. The connection should not be loose.
3. Safety devices for stable voltage should be installed on line.
4. On/off switch should not be used frequently.
5. In refrigerators nothing should be kept in front of cabinet fan to allow free circulation of air.
6. These should not be opened frequently/unnecessarily. The door should be opened, only when required and that also for as brief a period as possible.
7. The compressor should be protected from water.
8. The coils and compressor should be cleaned periodically to keep these dust and moisture free.
9. Interior should be cleaned periodically and disinfected. Spills must be avoided.
10. Ice should be frequently removed from freezers and deep freezers by defrosting.

*p*H METER

A *p*H meter is a device to measure the *p*H of a solution. The *p*H is defined as negative logarithm of hydrogen ion concentration. It is the measure of acidity or alkalinity of the solution. A neutral solution has *p*H of 7.0 and contains equal number of hydrogen (H^+) and hydroxyl (OH^-) ions. An acidic solution has excess of H^+ ions and a *p*H less than 7.0. An alkaline solution has excess of OH^- ions and a *p*H of more than 7.0.



Principal

*p*H electrode is an ion selective electrode (ISE) consisting of a measuring and a reference electrode combined together in one probe. It is lowered into the solution. The potential difference between the measuring and reference electrode is a function of hydrogen ion activity (concentration) in that solution. This is translated into *p*H by the instrument and reading is displayed.

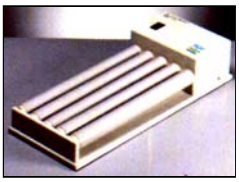
Precautions

All ion selective electrodes are very delicate and expensive. These require very careful handling. These should always be kept absolutely clean. Electrodes should not be allowed to dry up as this would cause permanent damage. They need to be kept always dipped in a buffer or a neutral solution when not in use.

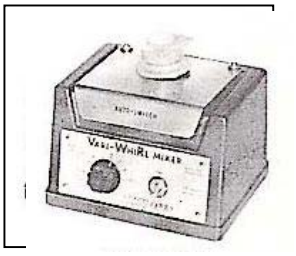
MIXERS

A mixer is a device used for mixing the contents of a tube or container. These are of following types:

1. **Roller mixer**: This comprises of Teflon coated cylinders set at horizontal plane with a gap just enough to allow their free axial movement. The cylinders are attached to rods at each end. Electrical motors allow movement of these cylinders in two planes. One rotator (axial) movement around the long axis of the cylinder, the other is a tilting movement in which one end of the cylinders goes up while the other goes down. This motion is continuously repeated. This allows thorough mixing of contents. This type of mixer is most commonly used to mix the biological fluids containing cellular components, particularly blood, before enumeration of cellular elements.



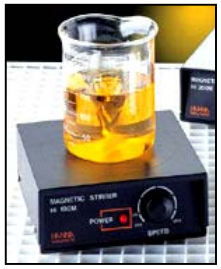
2. **Whirl mixer**: This equipment comprises a rapidly rotating rubber cup. When the bottom of a test tube is brought in contact with it, whirling movement is generated in the fluid contained in the tube. It permits thorough mixing, particularly when two fluids are to be mixed. It is commonly used for preparing serum dilutions and mixing of liquid reactants.



3. **Rotator mixer**: In this equipment a plate is rotated around its centre of gravity. The fluid drops on the plate get mixed without spreading much. It is mostly used for particle

agglutination tests e.g., VDRL.

4. **Magnetic mixer (stirrer)**: This equipment provides a magnetic force, moving in a circle under a plate. The container of solute containing solvent is placed on the plate. Iron pellets are then placed in the fluid. The circular movement of these pellets allows mixing of the contents.



5. **Shaking mixer (shaker)**: These equipment shake the tubes or containers placed in a stand fixed to it. These may be combined with a water bath to provide constant incubation while shaking.

Besides these various other special types of mixers are also available.

INCUBATORS

Incubators are used for maintenance of constant internal environments such as temperature, humidity and a particular gas concentration in a limited space called incubation chamber. The range of temperature, humidity and gas concentration vary in different incubators and are adjustable within limits. The incubators can be of cold type if the temperature is maintained below that the atmosphere outside. These are provided with refrigeration system (page 23). In this section are described hot incubators, which are used to maintain a temperature higher than that of atmosphere. Heating is achieved by hot air, water or oil. The source of heat is usually an electric element. A fan in case of hot air or a pump in case of hot water or oil is used to circulate these around the chamber. The whole system is insulated from outer atmosphere by a casing. The humidity is maintained by controlled heating of water at the base of incubation chamber. The gas concentration in the incubation chamber is controlled by flow of required gases from an external chamber through a regulator. The incubation chamber is usually divided into convenient spaces by adjustable, perforated metal shelves. The chamber may be provided with a glass door to provide additional protection against leak of atmospheric conditions. It is also provided with sensors for temperature, humidity and gas concentration that are connected to display and adjustment knobs or buttons on the exterior controlled by appropriate microprocessors. In modern incubators the displays are of digital type. Some incubators are provided with an alarm system that sounds if the atmospheric conditions in the chamber deviate from the pre-set values.

COMMON TYPES

1. **Simple incubator**: These regulate only the temperature in the incubation chamber. These are the most commonly used incubators in the laboratory for bacterial cultures and incubation of other materials.
2. **Anaerobic incubators**: In these incubators oxygen inside the chamber is replaced with nitrogen to provide an anaerobic atmosphere. These are commonly used to culture anaerobic microorganisms.
3. **CO_2 incubators**: In these incubators the air inside the incubation chamber is replaced with a mixture of 5-10% CO_2 in air. This can be achieved by release of CO_2 in the chamber or by release of the required mixture. These are commonly used to culture some microorganisms and tissue cells (page 169).
4. **Cell Culture incubators**: These are the most sophisticated incubators. Whole atmosphere inside the incubation chamber is controlled to provide precise temperature, humidity and CO_2 concentration. These are used for culture of cells and tissues.

Uses

1. Incubation of bacterial, cell and tissue cultures as well as biological reactants as in Widal and Coomb's test etc.
2. Slow evaporation and drying of salts etc. (only dry incubators are used).

Precautions and maintenance

1. The incubators should be cleaned and disinfected periodically by washing with suitable detergent, antiseptic solution and finally with alcohol.
3. These should not be opened unnecessarily.
4. Atmospheric conditions inside the incubation chamber need to be checked periodically by placing appropriate sensors.
5. The attached gas cylinder (if any) needs to be checked daily for the remaining gas.

OVENS

An oven provides a temperature higher than that of atmosphere. These are similar to incubators in mechanism except that much higher temperatures can be achieved in contrast to

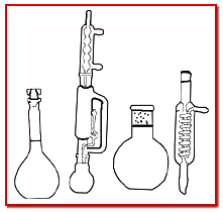
incubators. The temperature range covered by incubators is usually between 10-70°C, whereas in ovens it is between 50-250°C. These are used for rapid evaporation of materials, rapid drying and for sterilisation of articles that can be sterilised by dry heat.

WATER STILL

Water stills are used for distillation of water in the laboratory. Distilled water is use for washing of glassware, preparation of reagents, media and reactants. Distillation is a process in which water is heated to generate steam, which is then condensed to pure water by rapid cooling. The water thus produced is not only sterile but is free of all contaminants except volatile impurities which themselves evaporate at high temperature.

COMPONENTS AND OPERATION

1. **Heating chamber**: In this chamber water enters through an inflow pipe attached to a tap. Water is then heated with an electric element or by a gas burner placed underneath to generate steam. The chamber is also provided with an outflow pipe to drain off left over water and cleaning.



2. **Condenser**: The steam is passed to condenser through an outlet. The condenser is a double jacket pipe. Steam in inner jacket is cooled by continuously flowing cold water in the outer jacket. The condensed water from inner jacket is collected in suitable containers.

Types

There are two types of stills; 1) Metallic or stainless steel stills and, 2) All glass stills.

Precautions and maintenance

1. Periodic cleaning is required to remove the deposit in the heating chamber.
2. Heating chamber should not be left filled with water when not in use.
3. Quality of distilled water produced should be checked periodically to ensure that high quality water is being prepared.

Limitations

1. Only non-volatile substances are removed from water by the process of distillation.
2. Non-volatile substances left in the heating chamber may corrode the chamber.
3. Due to boiling and agitation of water some raw water may spill over into the condenser and contaminate the distilled water. This should be taken care of by maintaining the upper level of raw water in chamber at appropriate level.

Qualities of distilled water

Distilled water is colourless, sterile, free from non-volatile impurities and safe for preparation of most of the laboratory reagents. It causes little interference in chemical tests. The quality can further be improved by re-distilling the distilled water once (double distilled water) or twice (triple distilled water). This can be achieved either in stepwise manner or by attaching two or three stills in a row. In later case water from condenser of one still is directly collected into the heating chamber of next still in a sequence.

DEIONISER

Deioniser removes all ionic impurities from the raw water. In this process raw water passes through columns packed with ion exchange resins. These resins contain both positively charged and negatively charged radicals, which adsorb opposite ions from the water flowing pass the resin. There are two types of resins; anion exchange resin which attracts anions (or positively charged ions) and cation exchange resin which attaches cations (negatively charged ions) by electrostatic force. These resins are kept in cylinders separately or mixed together in the form of columns. Depending on the arrangement there may be one, tow or three cylinders connected in a sequence. In a three column system water to be deionised flows through cation exchange resin, anion exchange resin and finally into third column which contains a mixture of both resins (mixed bed). From here the water is collected into a suitable container though an outlet pipe. Both the resins are placed in separate cylinders in two cylinder systems, whereas a single cylinder system consists of mixed resin container.

Precautions and maintenance

1. The impurities that are introduced into deioniser with water limit the life of resins. Try to use as clean water as possible. It is better to use distilled water.
2. The quality of water produced should be checked periodically. The resins should be replaced or re-charged when the quality starts deteriorating.

Limitations

Deionisation does not remove organic chemicals, particulate matter and

microorganisms.

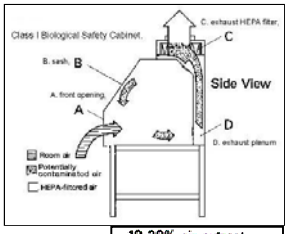
Uses

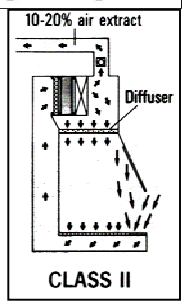
1. Deionised water is used in estimation of ionic materials e.g., sodium, potassium, lithium, calcium, magnesium, iron etc.
2. It is used to prepare culture media and reagents where ionic contamination may alter the conditions of the experiment.

SAFETY CABINETS

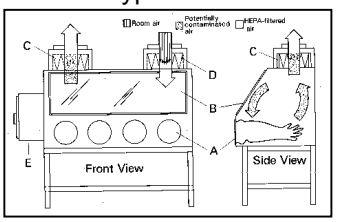
Safety cabinets are used in the laboratory for procedures in which either the reactants are to be protected from contamination by the worker or environment, the worker needs protection and safety during handling of infectious material or both of these are to be protected form each other. Based on the requirement, various types of safety cabinets are available.

1. **Laminar flow clean-air safety cabinet**: In this type of cabinet, air is first purified from particulate matter including microorganisms, with the help of a blower by passage through a HEPA filter under pressure. This clean air then flows, in laminar fashion, through the cabinet and to outwards from the operating front. This protects the work being carried out in the cabinet from contamination with microorganisms but cannot protect the worker from infectious material if handled inside the cabinet. Therefore, this type of cabinet is used for clean work, such as preparation of media, putting up tissue/cell cultures and handling sterile tissue. It is not suitable for handling infectious material.
2. **Class-I Microbiological safety Cabinet**: In this type of safety cabinet the air is drawn from outside into the cabinet from operating front and then it is passed through a HEPA filter and blown out with a blower fan. Air flows across the front panel or opening around worker into the cabinet. Therefore, it protects the worker and the atmosphere from exposure or contamination from the material being handled in the cabinet. This is used to handle infectious material.





3. **Class-II Microbiological safety Cabinet**: In this type of cabinet air is blown into the cabinet from the top after purification by passing it through a HEPA filter. The purified air flows towards base of the cabinet from where it is blown out of the cabinet, again after purification by another HEPA filter. The pressure of air inside the cabinet does not allow air from the atmosphere to enter the cabinet through the open operating front. This type of cabinet protects operator, material inside the cabinet and atmosphere. This is most commonly used safety cabinet in experimenting with contagious material.
4. **Class-III Microbiological Safety Cabinet**: The basic design of this type of cabinet is the same as that of Class-II cabinet except that:



   a. Operating front is closed with a curtain which has in-built gloves to handle the material placed inside the cabinet.
   b. Multiple HEPA filters are provided both at the inflow and outflow of air.

   c. This cabinet provides maximal protection to the operator, material and the environment. This type of cabinet is used when highly infectious/contagious material is to be dealt with.

Most of the cabinets are provided with ultra-violet lamps inside the cabinet. These can be switched on for additional sterilisation when required.

Precautions and maintenance

1. All the material required for the experiment or procedure to be carried out should be placed inside the cabinet before starting the actual work.
2. The cabinet shall then be switched on. If UV light is required it should also be switched on.
3. The operator must wear all personal protective equipment even when using the safety cabinet.
4. At the end of the work session, all articles should be removed from the cabinet. UV lamp and the fans should be switched off.
5. The cabinet should then be thoroughly cleaned, first with suitable detergent and then with a disinfectant. It should be wiped

dry and closed. Never leave the cabinet open without cleaning.

6. Particular attention should be paid to the perforated base and the space underneath while cleaning.
7. HEPA filters must be replaced as advised by the manufacturer or whenever found damaged or clogged.
8. UV lamp, blowers etc. should be checked and serviced regularly.

4. LABORATORY GLASS AND PLASTIC WARE

TYPES OF GLASS

Following are the types of glass commonly used to make laboratory glassware.

1. **High Thermal Resistant Glass**: Borosilicate glass with low alkali is a type that is resistant to heat, corrosion and thermal shock. Most common example is **Pyrex**. It should be used whenever heating or sterilisation by heat is required. A superior variety is **Corex** that is a special aluminium silicate glass and is six times stronger than borosilicate glass.
2. **High Silica Glass**: It contains 96% silica and is made from borosilicate glass by removing all elements except silica. This heat stable glass is used in high precision analytical work. It can also be used in manufacturing of reflectors and mirrors.
3. **High Alkali Resistant Glass**: It is boron free glass with much less thermal resistance. It is often called soft glass. It must be heated and cooled very carefully. Its use should be limited to procedures where strong alkalis are to be used.
4. **Low Acting Glass**: It contains materials which usually impart an amber or red colour to the glass and reduce the amount of light transmitted to the substance in the glassware. It is used for keeping substances that are particularly sensitive to light such as silver nitrate.
5. **Standard flint Glass or Soda Lime Glass**: It is composed of mixture of oxides of silicon, calcium and sodium. It is the most inexpensive glass but is less resistant to high temperature or chemicals.

CLEANING OF GLASSWARE

All glassware for the laboratory must be washed and cleaned thoroughly. In most cases it must be cleaned chemically and in some cases it must be cleaned from microorganisms i.e., needs to be sterile. Glassware that cannot be cleaned immediately after use should be rinsed with tap water and left to soak in a basin to which a small amount of detergent is added. Never allow dirty glassware to dry out. New glassware is often slightly alkaline and should be soaked for several hours in a dilute hydrochloric or nitric acid solution and then washed in the usual manner.

General Cleaning Procedure

Most glassware (with the exception of pipettes) can be cleaned in the following way:

1. Put the specified amount of detergent into a dishpan containing moderately warm water.
2. Rinse glassware in tap water and then put it in detergent solution for at least one hour.
3. Using a cleaning brush, thoroughly scrub the glassware. Avoid use of abrasive cleaners.
4. Rinse the glassware under running tap water. Allow the water to run into each piece of glassware, pour it out and repeat several times (7-10). Rinse the glass from outside also.
5. Rinse with distilled water.
6. Glassware may be dried in hot air oven at 50-100°C or at room temperature. Always dry glassware or other equipment in an inverted position to ensure complete drainage of water as it dries.
7. Check the glassware for cleanliness by observing the water drainage. Chemically cleaned glassware will drain uniformly. Dirty glassware will leave water droplets adhering to the wall of the glassware.

Cleaning of Pipettes

1. Place the pipettes immediately after use in a special pipette container having water in it. The water should be enough to completely cover the pipettes.
2. Place them in cleaning solution (mixture of sulphuric acid and potassium dichromate[1]). Soak for 30 min (detergent solution may also be used).
3. Rinse thoroughly in tap water to remove traces of the cleaning solution.
4. Rinse in de-ionised water 2-3 times.
5. Dry in a hot air oven.

Cleaning Diluting Pipettes

1. Rinse immediately after use.
2. First clean with tap water, then with distilled water. Finally rinse with either alcohol or acetone.

Cleaning Photometry Cuvettes

1. Cuvettes must be scrupulously clean and free from grease, smudges or scratches.

[1] This is a highly corrosive mixture therefore, handle it very carefully as it may cause serious burns.

2. Immediately after use rinse with tap water and fill with mild detergent solution and place in a special test tube rack.
3. Rinse with tap water and finally with distilled water.
4. Dry in a medium hot oven (always less than 100°C).

PIPETTES

Pipettes are special type of long narrow tubes, open at both ends, which are used for fluid column measurements. Their upper end is wide which is used for applying suction pressure and lower end is tapering which is used for drawing in or releasing the fluid. They are calibrated to indicate the volume. They can be made of glass or plastic.

SIZE

Depending upon their size they are divided into macro pipettes that have a capacity of 1 ml or more and micropipettes that have a capacity up to 1 ml.

Macro pipettes
Two types of macro pipettes are usually used in clinical laboratory. These are transfer pipettes and graduated or measuring pipettes.

1. **Transfer Pipettes**: They are designed to deliver a fixed volume of liquid. They consist of a cylindrical barrel in the centre with narrow glass tubing at both ends. These pipettes are calibrated with mark at the upper suction end and lower tapering end. They are further divided into:
   a. Volumetric Transfer Pipettes are used to deliver a fixed volume of aqueous solution.
   b. Otswald Folin Pipettes are used for accurate measurements of viscous fluids such as blood or serum. They have their bulb close to the tapering end, so that the surface area of the pipette in contact with liquid can be reduced. They have an attached ring near the mouthpiece to indicate that they are blow out pipettes.
2. **Graduated or Measuring Pipettes**: These are drawn out towards their tips and are uniformly calibrated. They are again of two kinds.
   a. Mohr pipettes are calibrated between two marks on the stem.
   b. Serological pipettes have graduation marks down towards the tip. The latter are blow out type of pipettes (see below).

Micropipettes
These are used to deliver (TD) or to contain (TC) very small volumes of fluids up to 1 ml.

FUNCTIONAL TYPES

1. **To Deliver (TD)**: In this type the pipette when filled up to the upper mark contains that much volume of fluid. It is to be emptied by touching its end against the tube wall in order to deliver that much volume.
2. **To Blow out (B)**: In this type once the pipette fluid is drained the residual volume of fluid is blown out in order to deliver the required volume. These pipettes have an etched ring near the mouth end with the volume written below it.
3. **To Contain (TC)**: These pipettes have only one mark on their stem that indicates a specified volume that the pipette contains when filled to that mark. These must be blown to empty. Then the fluid in which the specimen is blown out should be sucked up and down to wash out the whole specimen. The best example is Sahli's Hb pipette.

QUALITY

The best quality pipettes are called type A pipettes. Others are named as type B, C, D and E respectively. Type D & E are poor quality pipettes.

CALIBRATION

Delivering the specified volume of mercury with the pipette into a pre-weighed clean glass beaker checks calibration. The beaker is weighed again. The weight of the mercury in mg should be in accordance with the volume in ml.

PRECAUTIONS FOR USE OF PIPETTES

1. Suction force should be applied with the help of a rubber bulb, teat or pipette filler attached to the suction end. **Mouth pipetting must not be done in any case**.
2. Once the fluid has been drawn in the pipette to the required level, suction force should be maintained so that fluid is not lost while transferring. If a rubber bulb is used the pressure should be maintained.
3. Fluid should be drawn to a slightly higher level than required and the upper end should be immediately covered with the pulp of index finger. Then the level of fluid is adjusted to the required volume by slight release of finger pressure.
4. For coloured fluids the level of upper meniscus is taken as the indicator of volume while for colourless fluids level of the lower

meniscus is taken.

AUTOMATIC PIPETTES

These pipettes comprise a body and a tip. The body contains a pre-calibrated piston system which when pressed and released sucks a precise amount of fluid in the tip. Disposable tips made of plastic are used and discarded after use. These pipettes are of two types. One type is prefixed for a single specified volume. In other type, the volume can be adjusted within a narrow range. Both the types are available in different volumes with different sizes of tips. These must be checked for their accuracy from time to time because with wearing of spring system their accuracy may be lost. They are best used when very small amounts of liquid are to be delivered very quickly and in precise amount.

AUTOMATIC DELIVERY SYSTEM

In place of pipette this system is available to directly siphon the required amount of fluid from a bottle to another container. The system is directly attached to the bottle containing reagent and adjusted to required volume. It is useful when same amount of same reagent is repeatedly used.

PASTEUR PIPETTE

Pasteur pipette is a piece of tube, one end of which is drawn to very narrow diameter and a rubber bulb is attached to the other end. This is used when a fluid is to be delivered in drops of specified volume. These are also called dropping pipettes or droppers and their stem can be graduated for volume indication. Disposable Pasteur pipettes made of plastic are also available. These are useful for handling infections material such as serum etc.

TEST TUBES

These are the most commonly used glassware in any laboratory. They are cylindrical in shape with one end closed and the other open. The closed end is called the bottom. Test tube may be conical in shape with a narrow conical bottom and these are often used for centrifugation. Both types of test tubes may be stoppered (with a glass or plastic cap) or non-stoppered. Both may be graduated or non- graduated. The quality of glass also varies according to their use. Test tubes are either made of glass or plastic. Plastic test tubes are usually disposable. In certain situations only plastic test tubes should be used e.g., for plasma and its dilutions in clotting tests. These however cannot be used where strong chemicals like acids are used and heating is required. The size of a test tube depends upon its volume. This varies from small precipitin tube that accommodates only 0.5-1.0 ml of fluid to large test tubes that can accommodate up to 200 ml of fluid. Most commonly used are three sizes. One with a volume of 2-3 ml for clotting tests and blood group serology. One with a volume of 5-7 ml (sugar tube) for most chemical tests and one with a volume of 10-15 ml mostly for centrifugation and filtration.

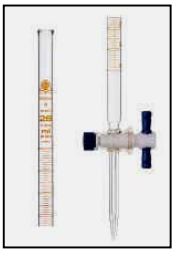
TEST TUBE STANDS

These can be made of wood, stainless steel or plastic. Their length and size varies according to the number and size of test tubes which these can hold. Where tubes are to be placed in an incubator or water bath steel stands should be used because wood and plastic are poor conductors of heat and the parts of the test tubes in contact with them remain cooler than the rest.

BURETTES

Burettes are modified types of glass pipettes designed to control delivery of a reagent drop by drop. These are usually used in titration.

SIZE



The sizes of burette vary form 1-100 ml or more. They are subdivided at different intervals depending upon the size of the burette. A burette having the capacity of 10 ml or less is known as micro burette.

SHAPE

They are wide bore glass pipettes in which the out flow of liquid is controlled by an all-glass or all-Teflon stopcock. All Teflon type does not require any lubricant while the all glass stopcock should be greased with petroleum jelly or similar inert lubricant. Some burettes have a reservoir and a 2-way stopcock for self-filling.

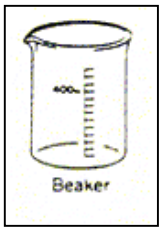
CALIBRATION

Burette calibration is verified by first filling the burette to a point just above the zero line with de-ionised water. Then the meniscus is very

carefully adjusted to the zero line. The drop of water hanging with the tip of burette is removed by touching it with the inside of a glass tube. The beaker is then weighed. Beaker is placed beneath the burette tip and the stopper is fully opened. When the fluid has dropped to about two cm above the last mark, the stopcock is closed. Then meniscus is gradually lowered to the desired volume and the last drop attached to the tip is removed by touching the glass wall of the beaker. The beaker is reweighed. It is checked that the desired volume in ml weighs correspondingly in mg after correction for temperature factor. Burettes for macro analysis have major graduation marks around the whole circumference of the burette and minor graduation marks at least half way round. This helps in minimising the errors in meniscus reading.

BEAKERS

A beaker is a glass or plastic container with a bottom and walls. The mouth is equal to its circumference and has a **beak** on one side. Beakers have many general uses and are made in different sizes varying from 10 ml to 5000 ml. Plastic beakers are usually resistant to most chemicals but cannot be used above 100°C. Different brands of beakers with different specifications are as under:



1. **Thick with slightly flared top spout**. These are excellent for pouring. Some are with strengthened rim with hair trimmed back accurate to ±5%.
2. **Heavy-duty** beakers have thick uniform walls with extra wall in top portion accurate to +5%. Used for mechanical washing and any hard use in laboratory.
3. **Beaker with glass handle** is ideal for handling hot solutions.
4. **Beaker with double spouts**. The double spout beakers are available with heavy walls. These are ideal for hot solutions.
5. **Heat resistant beake**r (withstand heat up to 900°C). These beakers are made of material containing 96% silica.
6. **Teflon beakers** are heat resistant to 260°C and are inert to all materials except molten alkaline metals.
7. **Polypropylene beakers** are resistant to chemicals and autoclaving. Polypropylene beakers are also available with handle and convenient pouring spout.
8. **Fleaker beaker**: Erlenmeyer flask that also serves as a beaker. Wide mouth eliminates spills. Narrow recessed neck reduces splash out during boiling or vigorous agitation. Autoclavable type is provided with polypropylene lid that keeps sample free of contamination.

FLASKS

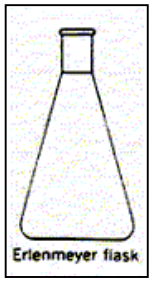
This is another important laboratory glassware. Flasks can be made of glass or plastic materials. Quality of glass also varies. There are two functional types of flasks.

GRAVIMETRIC

In these the volume adjustment is not so accurate. These are used for boiling, mixing, storage and preparation of reagents.

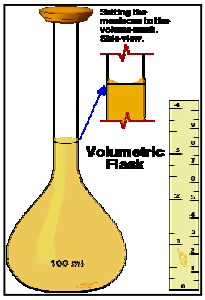
VOLUMETRIC

These are precisely calibrated for definite volume. These may be graduated or non-graduated. There are three types of flasks depending upon the shape.



1. **Conical flasks**: Their walls gradually narrow from bottom upwards but the mouth is still wide. Their bottom is flat and they can rest on their bottom.
2. **Round bottom flasks**: These are empty spheres of glass to which a wide mouthed short glass inlet is attached. These are to be held on a stand or with the help of a clamp attached to a stand. These are usually gravimetric.

3. **Volumetric long necked flasks**: These flasks have a flat, broad bottom and the walls narrow rapidly into a long narrow neck. The neck carries the calibration marks.



All flasks can be stoppered or non-stoppered and their sizes vary depending upon volume. These are commonly available in 10-1000 ml volumes.

CALIBRATION

Thoroughly clean the flask and dry. Weigh it accurately. Now fill it to the mark with de-ionised water adjusting the meniscus carefully. Meniscus can be adjusted with the help of a card that is half black and half white. This card is held one cm behind the flask neck in such a way

that top of black area is about one mm below the meniscus. The meniscus then appears as clearly defined thin black line. Now reweigh the flask and calculate the volume from weight of water after adjusting for temperature.

PRECAUTIONS FOR USE

1. Flasks must be absolutely clean. Filling with distilled water first and then emptying it can check this. Hold the flasks in inverted position so that all the water is drained. Now examine the walls for a thin film or droplets of water. These should not be there.
2. Chemical cleanliness is also important. Small amounts of detergent may be left behind. Washing the flasks with distilled water and checking *p*H of this water can check this. It should not differ from *p*H of water used for washing.
3. When measurements are made, meniscus must be correctly adjusted as described above.

CYLINDERS

These can be made of glass or plastic material. These are long and narrow having a mouth equal to their internal diameter. Mouth may not be beaked. Their body is graduated. They are used to measure approximate quantities of reagents or solutions. Their sizes depend upon the volume they measure and usually vary from 10 ml to five litres. While measuring fluids in a cylinder, precautions should be taken for adjustment of meniscus. These have been described earlier. One must see the meniscus with eyes parallel to its level.

MICROSCOPIC SLIDES

These are quadrangular pieces of thin, transparent glass of low refractivity. These are used for placing material on them for microscopy. The material can be fixed on it such as blood smears or mounted such as histological sections. Unfixed fluid material can also be placed on these as in case of urine and stool examination. A cover slip is required to spread it into a thin film.

CARE OF SLIDES

1. After use for unfixed material, slides should be soaked in a suitable antiseptic solution immediately.
2. Fixed slides and soaked slides are then left in detergent overnight.
3. They are rinsed in distilled water, wiped dry with lint free cloth and dried in an oven. They should be cooled before use.
4. Slides must be free from scratches.
5. Grease must be washed away from new slides.

COVER SLIPS

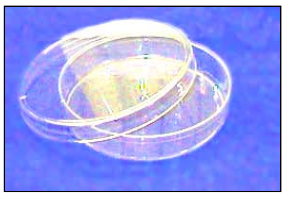
These are ultra thin rectangular pieces of transparent glass of good quality. These are used to cover the material placed on the slide for microscopy or to mount it permanently. These are available in different sizes. These should be used only once since they are difficult to clean.

PETRI DISHES

These are small containers to carry different types of media used for the growth of various microorganisms. These may be made of:

1. Glass (non disposable)
2. Plastic (disposable)

Glass petri dishes can be reused after proper cleaning and sterilising but it requires lot of efforts and is a time consuming process. Disposable plastic petri dishes are now available. They are discarded after single use. They are however costly. Besides these a number of other glassware e.g., desiccators, funnel, micro titre plates etc., are also used in the laboratory.



5. BASIC LABORATORY PROCEDURES

STERILISATION AND DISINFECTION

Microbiological work with pure cultures requires the use of culture media and containers, which are free from all live contaminating microorganisms. Two terms are used to describe the killing or removal of microorganisms. They are:

- Sterilisation
- Disinfection

STERILISATION

Sterilisation means the freeing of an article from all organisms, including viruses, bacteria and their spores, fungi and their spores, both pathogenic and non-pathogenic. It is an absolute germ-free state. Sterilisation is required for culture media, suspending fluids, reagents, containers and equipment used in the laboratory.

METHODS OF STERILISATION

Following main methods are used for sterilisation:

1. **Heat**: Heat is applied in its two forms i.e., dry heat and moist heat. It is very reliable and widely applicable method. Temperature above 100°C under controlled conditions kill spores as well.
2. **Ionising Radiation**: Beta (β, electrons) and Gamma (γ, photons) irradiation are used in the industry for disposable single use items like needles, syringes, latex catheters and surgical gloves.
3. **Filtration**: Used to remove bacteria from fluids, which are spoiled by heating e.g., blood, semen and antibiotic solutions.
4. **Chemical Disinfectants**: These can be:
   a. Gases: Ethylene oxide is used mainly in industry for sterilisation of heat sensitive material, which cannot withstand heating such as plastics.
   b. Liquids: Certain liquids such as glutaraldelydes can be used when no other sterilisation method is available. These are not very effective and reliable.

STERILISATION BY DRY HEAT

Dry heat is suitable for glassware, instruments, and paper-wrapped articles not spoiled by very high temperatures, and for water impermeable oils, waxes and powders. Dry heat cannot be used for water containing culture media. Methods of application of dry heat include:

1. **Red Heat**: The articles to be sterilised are put in the flame directly until red-hot. It has its application in the sterilisation of inoculating wires and loops, tips of needles and forceps, which should be held vertically in flame until red hot along their whole length.
2. **Flaming**: This means direct exposure of articles to gas or spirit flame. This method, however, does not ensure complete sterilisation.
3. **Hot air oven**: This mode of heat is applied for substances, which can withstand high temperatures in the range of 160-180°C and cannot be reliably penetrated by moist heat. It is used for glassware such as tubes, flasks, measuring cylinders, all glass syringes and glass pipettes, powders, oils and greases in sealed containers. Hot air oven is used for sterilisation of:
   a. Glassware
   b. Forceps, scalpels, scissors etc.
   c. Throat swabs
   d. Syringes
   e. Dry materials in sealed containers
   f. Powders, fats, oils and greases, which are impermeable to moisture.

   Following precautions should be observed when using hot air oven:
   a. The oven must not be over loaded. Space must be left for circulation of air through the articles.
   b. It must first be loaded and then heated up to the sterilisation temperature in the courses of 1-2 hours.
   c. A holding period of one hour at 160°C is required for sterilisation. It means one hour after attaining 160°C.
4. **Infrared radiation**: Infrared rays are generated by an electric element and these rays are allowed to fall on the objects to be sterilised. The object is heated and sterilised

STERILISATION BY MOIST HEAT

Methods of application of moist heat include:

1. **Pasteurisation**: This method is used for sterilisation of milk. Temperature required is either 63-66°C for 30 min or 72°C for 20 seconds. By this method eating utensils, clothes and bed sheets of patients can also be sterilised.
2. **Boiling**: Simple boiling is used for sterilising articles like syringes. Moist heat at 100°C continuously for 90 min is used to sterilise culture media. Intermittent exposure at 100°C for 20-30 min for three consecutive days is called **Tyndallisation**. This is used for materials, which are destroyed or denatured by prolonged heat such as media containing sugars. It allows killing of germinating spores.
3. **Steaming below 100°C**: Steaming below 100°C is used for delicate material.
4. **Steaming above 100°C**: Moist heat at this temperature is achieved using heat under pressure. The equipment used for this purpose is called an autoclave. Household pressure cooker is a good example of a simple autoclave.
5. **Steaming above 100°C under pressure**: This is most effective method of sterilisation and requires an autoclave.

AUTOCLAVE

Autoclave provides moist heat (steam) at temperatures above 100°C at greater than the atmospheric pressure. The superheated steam condenses on cooler load releasing thermal energy as well as moisture. Combined effect of both these is denaturation of microbial proteins. Majority of culture media are sterilised by autoclaving. This destroys the bacterial endospores as well as vegetative cells. It is important to sterilise a medium at the correct temperature and for the correct length of time (as instructed in the method of preparation). Under-autoclaving can result in an un-sterile medium, which will need to be discarded. Over-autoclaving can cause precipitation, alteration of *p*H and the destruction of essential components in a medium.

Principle

Water boils at 100°C. At this temperature vapour pressure equals the pressure of the surrounding atmosphere i.e., 760 mm Hg or 14.7 pounds per square inch (psi) or 016 (one bar) in pressure gauge. When water is heated within a closed vessel, the pressure inside increases with a corresponding rise in the boiling point of the water. The steam thus formed is superheated, much above 100°C. Thus in an autoclave the articles are exposed to moist heat at higher temperatures than 100°C.

Uses

Autoclave is used to sterilise surgical supplies (instruments), linen and most of the bacteriological culture media.

Precautions

1. All parts of the load must be permeated by steam; therefore, load should be loosely arranged.
2. Steam should be saturated and dry.
3. There is a minimum holding time for various temperatures and pressures necessary for complete sterilisation (Table 5.1).

Table 5.1: Holding time at various pressures.

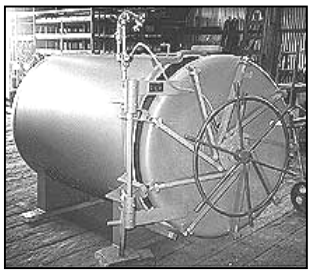
Steam Pressure (lU/Square Inch)	Temperature (°C)	Holding Time (min)
0	100	-
10	115	45
15	121	18
30	134	03

4. Air must be removed completely from the autoclave chamber and from the load so that the load is subjected to pure steam during the process of autoclaving.

Types

1. Simple non-jacketed: This is also called the pressure cooker type of autoclave. It has a vertical or horizontal cylinder of metal, usually stainless steel, in a supporting frame or case. The cylinder contains water up to a certain level and a gas burner or electric heater below the cylinder heats this. The lid is fastened by screw clamps and made airtight by asbestos gasket. At the top of the autoclave there is a discharge tap, pressure gauge and a safety valve. The discharge tap is kept open for a few min after the water begins to boil to allow all the air in the chamber to escape. When steam starts coming out the tap is closed. The pressure starts rising till it reaches the desired level. At this the holding period begins. Temperature is maintained for the desired length of time. Heating is then stopped, the pressure on the gauge starts falling to atmospheric pressure. Autoclave is then cautiously opened. If it is opened while still under positive pressure, a serious explosion may occur. It has few drawbacks:
   a. The method of discharging air is inefficient.
   b. It lacks the mechanism of drying the load after sterilisation.
2. Steam jacketed autoclaves with automatic air discharge: These consist of a horizontal

or vertical metal cylinder to which a door is fastened by a capstan head that operates by bolts and automatically remains locked while the chamber pressure is raised. It has a supply of steam from an external source. It has a steam jacket that heats the sidewalls independent of the presence of steam in the chamber and thus dries the load. There is a channel and a thermostatic valve to control the discharge of air automatically. A thermometer is fitted to show the temperature in the discharge channel above the no return valve. This is the temperature of the lowest and coolest part of the chamber. A vacuum system is provided to assist in drying of the load. An air intake channel with self-sterilising filter for introducing warm sterile air into the chamber is present.



3. High Pre-Vacuum Steriliser: They are equipped with electrically driven pumps, which produce a vacuum in the chamber. This allows the steam to penetrate very rapidly.

Operation

1. Steam is first introduced into the jacket, which is kept filled throughout the day at a temperature of 121°C.
2. When the jacket is hot the load is placed in the chamber.
3. The door is closed and steam is allowed to enter the chamber.
4. The air and condensate start coming out of the discharge channel. When all the cool air is discharged and pure steam starts coming out, a temperature of 121°C is reached and the steam trap is automatically closed.
5. Now the holding period starts which differs for different articles.
6. At the end of the holding period the supply of steam to the chamber is stopped while that to the jacket is maintained. The steam left in the chamber begins to cool by loosing heat and hence the pressure starts falling.

Controls and Indicators

1. Physical control

a. Automatic Process Control: This control system carries through the whole sterilising cycle according to a pre-selected scheme for the duration, temperature and pressure of each stage. After the chamber is loaded and automatic system is started no further step is required until the load is ready for removal. Monitoring system ensures that if the temperature at any time falls below the selected one the operation will be repeated.

b. Recording Thermometer: This makes a graphic time record of the temperature changes in the discharge channel and hence helps the operator to avoid errors in timing and holding period.

c. Thermocouple Measurement of Load Temperature: This method is used for finding the heating up time for a given kind of load. A thermocouple is inserted deep inside an article in the autoclave chamber; its leads are carried out under the channel door and connected to the potentiometer. It indicates the temperature inside the test article during autoclaving.

2. Chemical control

a. Browne's control tube: It contains red solution, which turns green when heated at 121°C for 25 min. It must be stored below 20°C to avoid deterioration and premature colour change.

b. Bowie Dick tape: This adhesive tape also works on the same principle. There are printed lines on the tape, which turn black when appropriate temperature is achieved (121°C).

3. Biological control

Spore indicator: A preparation of bacterial spores is placed within the load in the autoclave and is tested for viability after autoclaving. *Bacillus stereothermophillus* requires to be, cultivated at 55-66°C and its spores are killed at 121°C in about 12 min. The various commercial forms of such spores are available. The spores are placed on strips and after the autoclave load they are to be cultured. In other form the spores are present in ampoules and the fluid changes colour if the recommended temperature is achieved so the organism has not to be cultured.

STERILISATION BY FILTRATION

Different filters are used to make the solutions and fluids bacteria free. Filtration is used for those materials, which are destroyed by heat e.g., antisera, and toxins. There are two types of filtration:

- Surface filtration
- Depth filtration

Surface Filtration
In this type of filtration, particles having larger diameter than the pores of the medium are retained on the surface of the medium and filtrate passes through the pores. It is performed with:

- Filter papers
- Membranes
- Sieves

FILTER PAPERS

Filter papers are specially made papers with specific porosity, speed of filtration and retention to meet the needs of qualitative and quantitative analysis. Generally filter papers are divided into two main categories:

a. Qualitative papers (ash content not more than 0.06%).
b. Quantitative papers (ash content less than 0.01%).

The above two classes are further divided into three subclasses according to their porosity and speed of filtration.

Quantitative Ash-less papers

1. Papers having rapid speed, smooth texture and coarse porosity are suitable for coarse and gelatinous precipitates, which require thorough washing on paper.
2. Medium speed, medium porosity papers with smooth texture are suitable for general gravimetric analysis.
3. Slow speed, fine porosity papers of smooth dense texture are suitable for vacuum filtration (Table 5.2).

Table 5.2: Types of filter papers at various speeds of filtration.

FILTRATION SPEED	POROSITY	QUALITATIVE PAPER GRADE	QUANTITATIVE PAPER GRADE
Rapid	Coarse	Whatman–4	Whatman–41
	Medium coarse	Whatman–SG	Whatman–54
		Whatman–1	
Medium	Medium	Whatman–2	Whatman–40
	Medium fine	Whatman–3	Whatman–44
		Whatman–5	Whatman–50
Slow	Fine		Whatman–42

Qualitative Ash-less Papers

1. Rapid speed, coarse porosity and smooth open texture papers are suitable for coarse and gelatinous precipitates. These retain hydroxides of iron and aluminium and metallic sulphides. These are good for clarifying solutions and oils and are widely used for sugar analysis.
2. Medium speed, medium porosity, smooth papers are suitable for clinical testing, clarifying pharmaceuticals and spot test etc.
3. Fine porosity, slow speed smooth dense texture papers are suitable for filtering finest particles or precipitates. These can be used with vacuum (Table 5.2).

MEMBRANES

These are made up of homogenous polymeric material such as cellulose acetate, cellulose esters, polyvinyl chloride (PVC) etc. Most commonly used are cellulose acetate and cellulose fibres. Pores occupy 80% of their surface area. Their basic structure is hydrophobic. These may be used under vacuum with positive pressure, with gravity in auto-analysers and in ultra-filtration to concentrate macromolecules such as proteins.

Depth Filtration
These filters are made up of cotton, fibreglass or asbestos. In this type of filtration particles are retained in the body as well as on the surface of the filter. In depth filters the matrix of fibres is usually arranged in random manner and they retain large particles. Different types of such filters are:

1. Earthenware e.g., Berkfield & Chamberland
2. Asbestos (Seitz)
3. Sintered glass
4. Cellulose membrane

DISINFECTION

Disinfection implies killing of vegetative forms of bacteria, viruses, fungi and parasites but does not completely eliminate spores and other non-vegetative forms. Disinfectants are chemical agents capable of disinfection. They kill microorganisms and occasionally spore, by the destruction of proteins, lipids or nucleic acids in the cell or its cytoplasmic membrane. They are used to decontaminate surfaces that have been in contact with body fluids, tissues etc, pathological specimens or microbiological cultures. These are divided into two broad groups:

1. **Antiseptics**: These are substances, which are non-toxic for living tissue and hence are used for skin disinfection e.g., spirit, alcohol, povidone, iodine etc. Antiseptics are basically the same chemicals as are disinfectants. It is their reduced concentration, which cause them to be used on human skin as less irritant e.g., 70% alcohol or 2% tincture of iodine
2. **Disinfectants**: They are strong chemicals and are used to disinfect nonliving objects. They are generally toxic and corrosive for the living tissues.

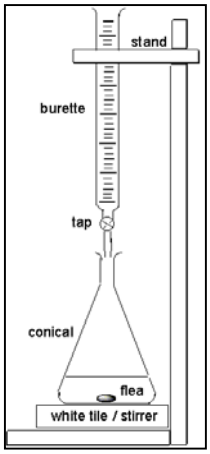
Types

1. **Phenolic compounds**: Phenol, Lysol, Cresol, Dettol, Phisohex, and Chlorhexidine

are used for decontamination of infective discharges, floors, bathrooms and bedpans.

2. **Halogen compounds**: Chlorine is used for water and food disinfection. Examples are Milton and Eusol. Iodine and Tincture Iodine are used for skin disinfection before surgery. Betadine or povidone iodine is for skin disinfectant and is very effective.
3. **Metallic Salts**: Mercuric chloride was previously used as skin disinfectant. Silver nitrate 1% is used as eye drops in the newborn for prevention of gonococcal eye infection.
4. **Formaldehyde**: It is a rapid bactericidal disinfectant and also kills bacterial spores. In liquid form, 10% solution is used as fixative and preservative for biopsy specimens for histopathology. It is used to sterilise instruments like cystoscope, laparoscope. In gaseous form it is used to disinfect rooms and articles, which are damaged by heat like bedclothes, blankets, respirators and catheters.
5. **Volatile Solvents***:* Ethyl alcohol as a 70% solution is used as skin disinfectant before giving injections. Acetone and ether are weaker than 70% alcohol as skin disinfectants.
6. **Soaps and Detergents**: These include soap and Cetavelon and are multipurpose disinfectants.
7. **Gaseous Disinfectants**: Formaldehyde gas has already been mentioned. Ethylene oxide can be used in place of formaldehyde gas.
8. **Miscellaneous**: Gentian violet is used as a mouth and skin paint for *Candida* spp infection. Potassium permanganate is used for disinfecting water and vegetables.

TITRATION



It is a procedure used to find out the concentration of an acid or base in a solution by reacting or neutralising it with a standard solution in a controlled manner with the help of an indicator. It is required in clinical chemistry for the estimation of normality of acids or bases in body fluids such as HCl in gastric juice.

Requirements

- Acid or alkali of known normality
- Indicator: Any chemical capable of changing its colour with change in *p*H such as phenolphthalein.
- Burette
- Beaker/flask
- Pipette

Procedure

For estimating normality of an acid (N_1) in a solution proceed as follows:

1. Pour a measured volume (V_1) of unknown solution in a beaker.
2. Add a few drops of 1% phenolphthalein indicator (page 48).
3. Fill the burette up to the zero mark with an alkaline solution of known normality (N_2).
4. Mix the alkaline solution drop by drop in the beaker till the faint pink colour of phenolphthalein indicator persists even after thorough mixing.
5. Note the volume of alkali used (V_2) to titrate the acid in the beaker.

Calculation[1]

Calculation of unknown normality of acid is done by the following formula:

$$V_1 \times N_1 = V_2 \times N_2$$

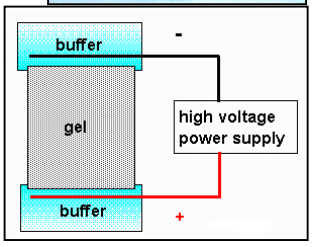
$$N_1 = \frac{V_2}{V_1} \times N_2$$

Where

N_1 = Normality of acid solution (unknown)
V_1 = Volume of acid solution
N_2 = Normality of alkali
V_2 = Volume of alkali used

ELECTROPHORESIS

INTRODUCTION



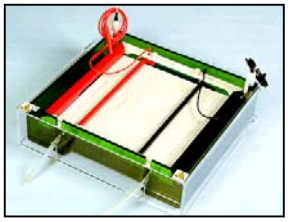
Electrophoresis is a technique based on the mobility of ions in an electric field. Positively charged ions migrate towards a negative electrode (cathode) and negatively charged ions migrate toward a positive electrode (anode). For safety reasons one electrode is usually at ground and the other is biased positively or negatively. Ions have different migration rates and can therefore be separated. Mobility of a particle is directly proportional to the voltage applied and the net charge of a particle, while it

[1] To find out the unknown normality of a base in solution, it is titrated with acid of known normality.

is inversely proportional to the friction offered by the particle in electric field depending upon molecular size and shape.

INSTRUMENTATION

The apparatus consists of a high-voltage supply, electrodes, buffer, and a support for the buffer such as filter paper, cellulose acetate strips, polyacrylamide gel, or a capillary tube. Open capillary tubes are used for many types of samples and the other supports are usually used for biological samples such as protein mixtures or DNA fragments. After a separation is completed the support is stained to visualise the separated components. Resolution can be greatly improved using ***isoelectric focusing***. In this technique the support gel maintains a *p*H gradient. As a protein migrates down the gel, it reaches a *p*H that is equal to its isoelectric point. At this *p*H the protein is neutral and no longer migrates, i.e., it is focused into a sharp band on the gel at that point.



Media for Electrophoresis

1. Paper (obsolete)
2. Cellulose acetate membrane (CAM).
3. Gels
   a. Starch Gel
   b. Polyacrylamide Gel (PAGE)
   c. Agar Gel
   d. Agarose Gel

CELLULOSE ACETATE MEMBRANE (CAM) ELECTROPHORESIS

Apparatus

An electrophoresis chamber or tank consists of two compartments separated by a partition. Each compartment has an electrode made of an inert material such as platinum. Each side is filled with equal amount of a suitable buffer solution. A bridge across the top of the partition holds a membrane or gel with each end of it in contact with the buffer directly or through paper wicks. The only connection between the two compartments is through this membrane. Sample is applied on to the membrane. Electrical power source is attached to the tank, which has an indicator for polarity. Current of prescribed voltage is applied. Molecules start migrating through the membrane to anode or cathode depending upon their net charge. After the prescribed time, current is switched off and the membrane or gel is removed from the tank. It is then treated with suitable fixative and is stained to make the separated bands visible.

Reagents

1. Cellulose acetate strips of suitable size
2. Barbitone buffer, *p*H 8.6, ionic strength 0.05. Dissolve 10.16g sodium barbitone and 1.84 g diethylbarbituric acid in about 800 ml water and make up to 1L.
3. Fixative solution is prepared by dissolving 5 g trichloracetic acid (TCA), 5 g zinc sulphate ($ZnSO_4$) and 0.35 g sulphosalicylic acid (SSA) per 100 ml distilled water.
4. Ponceau S, 0.5% w/v in 5% trichloracetic acid. Other protein stains such as commassie brilliant blue (CBB) or amido black can also be used.
5. Acetic acid, 5% v/v in water as destaining solution.
6. Clearing solution is prepared by adding 15 ml glacial acetic acid in 85 ml methanol. This solution is corrosive and volatile, therefore minimum amount needed should be prepared with precautions.

Procedure

1. The cellulose acetate strips are marked with lead pencil and soaked in running buffer in a shallow tray avoiding inclusion of air bubbles under the surface.
2. Soaked strips are lightly blotted to remove excess buffer.
3. The strips are placed over the bridge or supports in the tank and wicks of filter paper are placed over both ends to dip into the buffer.
4. From 3-5 µl sample is applied near the cathode in a row leaving spaces in between and clear margin on either side. Replace lid and connect power supply.
5. The current is adjusted to 0.4 mA per cm width of strip (~185V). Run for 20-60 min. Time and voltage or current varies with different apparatuses and procedures. After completion of electrophoresis, the strip is removed, trimmed and soaked for 5-10 min in a fixative solution (10% TCA).
6. Strip is then stained by submersion in Ponceau S solution for 10 min.
7. De-stained in several changes of acetic acid.
8. For densitometry, the strip may be used as such or it may be cleared by a dip in clearing solution and drying in an oven at 60-80°C.

Uses

1. Identification of abnormal patterns of plasma proteins in various disease processes

(Figure 5.1).

2. Identification/quantitation of normal and abnormal protein bands (Figure 5.2).
3. Identification and quantitation of normal and abnormal haemoglobins[1] (page 283).
4. Quantitation of lipoproteins.
5. Identification of isoenzymes.

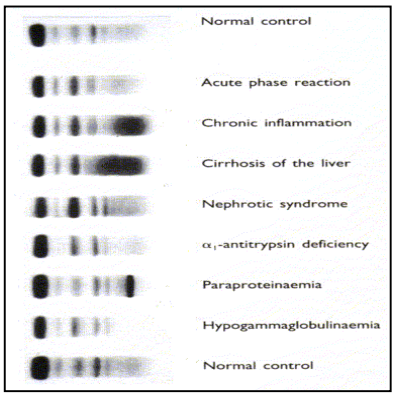


Figure 5.1: Patterns of serum protein electrophoresis in various diseases

SDS-PAGE

It stands for sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and is useful for molecular weight analysis of proteins. SDS is a detergent that dissociates and unfolds oligomeric proteins into its subunits. The SDS binds to the polypeptides to form complexes with fairly constant charge to mass ratios. The electrophoretic migration rate through a gel is, therefore, determined only by the size of the complexes. Molecular weights are determined by simultaneously running marker proteins of known molecular weight.

CHROMATOGRAPHY

It is an important technique for separating pure substances from mixtures. The chromatographic system consists of two immiscible phases, a stationary phase, which is fixed and granular, and a mobile phase, which flows through the interstices of the stationary phase. The mobile phase is fluid (or liquid or gas), and its movement is effected by gravity, applied pressure, or capillarity. The stationary phase is usually a finely divided insoluble solid. Chromatographic separation depends on the fact that different substances follow the moving solvent at different rates. Those substances whose distribution favours the moving phase pass more rapidly through the chromatogram than those, which favour the stationary phase.

[1] Reagents and procedure for haemoglobin electrophoresis vary from those given above.

There are two main types of chromatography (1) column chromatography, in which liquid passes down through particles of solid packed in a glass, plastic or stainless steel tube; (2) thin-layer chromatography, where the stationary phase is in the form of a very thin layer of silica gel or cellulose made to adhere to a glass plate or plastic sheet, up which the solvent moves by capillarity.

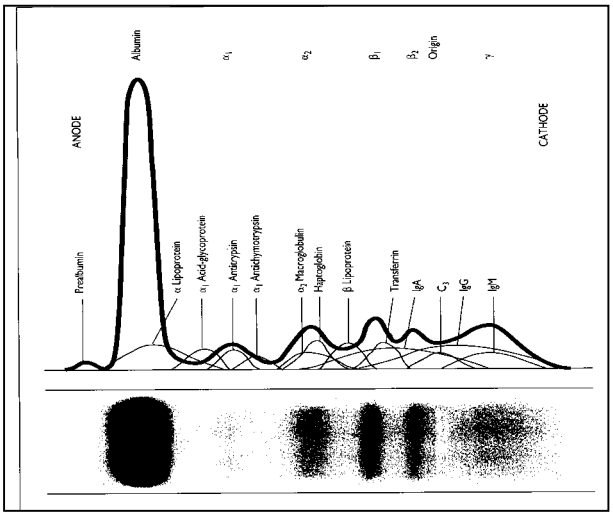
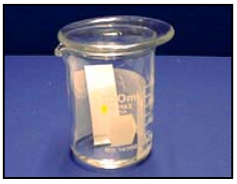
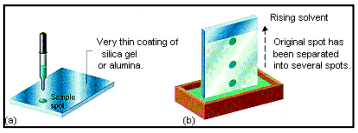


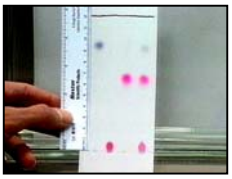
Figure 5.2: CAM electrophoresis of serum proteins and densitometric analysis

THIN LAYER CHROMATOGRAPHY

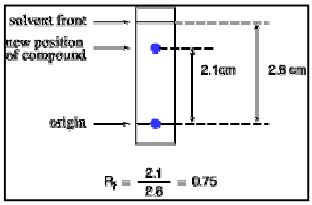


The thin-layer chromatography TLC) is a powerful, simple, inexpensive, rapid and versatile technique for separating organic compounds. It has found great use in clinical laboratory in the separation of amino acids and sugars in a biological solution such as urine or plasma. It consists of a stationary phase (silica, cellulose, alumina) bound to a glass or plastic plate with the addition of a binder (such as starch). The mobile phase is usually a solvent. The sample, either a liquid or dissolved in a volatile solvent, is deposited or applied as a spot on one edge of stationary phase. The bottom edge of the plate is then placed in a solvent reservoir and the solvent moves up the plate by capillary action. When the solvent front reaches the upper edge of stationary phase the plate is removed





from the tank. The plate is dried, and the area occupied by the separated components or spots is either visualised by ultraviolet light or is **developed** by placing it in iodine vapour, or by spraying the surface with a chemical that reacts with that component, e.g., Ninhydrin turns purple with amino acids, and sugar molecules react with resorcinol. Each component moves at a specific rate along the stationary phase so the components are separated. The unknown



constituents of the sample can be identified by simultaneously running a series of standards in parallel with the unknown components. The ratio of the distance travelled by any component to the distance travelled by the solvent is called Rf value, which remains constant for that component under the conditions of the test. Thus, their Rf values can be compared. In this way an unknown component can be identified. The plate can be run in one axis (one dimensional) or it may be run in two axes (two dimensional) thin layer chromatography. (see also THIN LAYER CHROMATOGRAPHY on page 375).

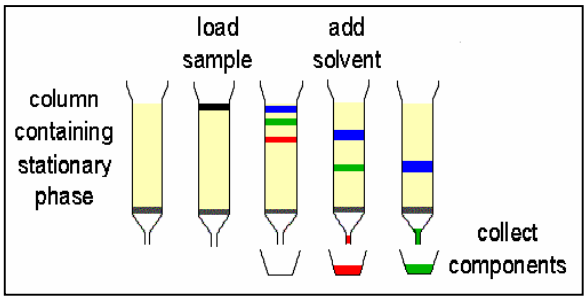
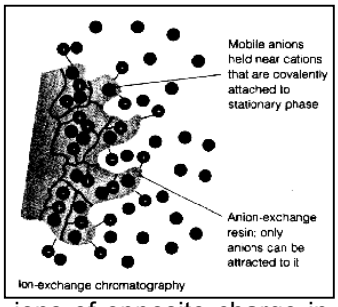


Figure 5.3: Schematic diagram of liquid chromatography

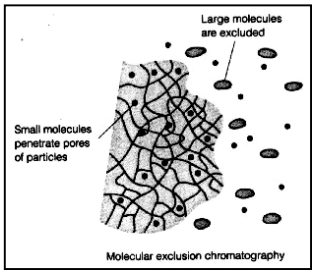
LIQUID CHROMATOGRAPHY

Liquid chromatography is an analytical technique that is useful for separating ions or molecules that are dissolved in a solvent. If the sample solution is in contact with a second solid or liquid phase, the different solutes will interact with the other phase to differing degrees. These differences allow the mixture components to be separated from each other. Simple liquid chromatography consists of column with a fritted bottom that holds a stationary phase in equilibrium with a solvent. The mixture to be separated is loaded onto the top of the column followed by more solvent. The different components in the sample mixture pass through the column at different rates due to differences in their portioning behaviour between the mobile phase and the stationary phase. The compounds are separated by collecting aliquots of the column effluent as a function of time. For certain applications pre-filled disposable small columns are available. It is used to separate and purify the individual components of a solution containing a mixture. Depending upon the solid phase it has following types:

1. **Ion-exchange chromatography**: In this type of chromatography a cellulose resin is packed into the column to which proteins and other molecules are covalently bound in varying degree by electrostatic forces. The resin can be anionic or cationic so to attract the solute ions of opposite charge in the mobile liquid phase.

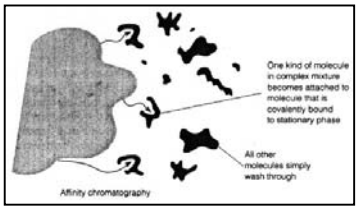


2. **Gel filtration, gel permeation or molecular exclusion chromatography**: The diffusion of small molecules into the pores of a gel from which large molecules are excluded because of their size forms the basis. This type of chromatography lacks an attractive interaction between the stationary phase and solute. The mobile phase passes through a porous gel, which separates the molecules according to their size. The pores are normally small and exclude the larger solute molecules, but allow smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at a faster rate than the smaller ones. The original starch has now been replaced by standardised cross-linked dextran known as Sephadex. Many grades are available and vary in the degree of cross linkage-this determines the upper limit of size of molecule that can enter the pores. Gel forming beads are allowed to swell in water and are then packed in the column. The method has found great use in the separation and purification of proteins; in general they appear in the column eluent in

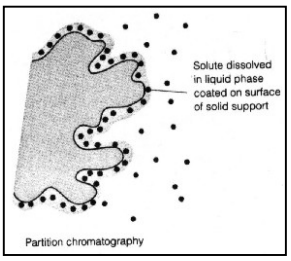


order of decreasing molecular size.

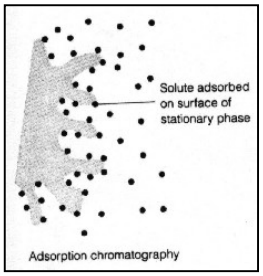
3. **Affinity Chromatography**: This is the most selective type of chromatography. It utilises the specific interaction between one kind of solute molecule and a second molecule that is immobilised on a stationary phase. For example, the immobilised molecule may be an antibody to some specific protein. When solutes containing a mixture of proteins are passed by this molecule, only the specific protein is reacted to this antibody, binding it to the stationary phase. This protein is later extracted by changing the ionic strength or pH.



4. **Partition Chromatography**: This form of chromatography is based on a thin film formed on the surface of a solid support by a liquid stationary phase. Solute equilibrates between the mobile phase and the stationary liquid.



5. **Adsorption Chromatography**: Adsorption chromatography utilises a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase. The equilibration between the mobile and stationary phase accounts for the separation of different solutes.



HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

This is a form of column chromatography where a mixture dissolved in liquid mobile phase is forced to flow through the stationary phase (a resin packed column) under pressure to resolve into components. These separated components are then passed through a detector and a chromatogram is generated. The instrument consists of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector. Different components of the mixture pass through the column at different rates due to differences in their distribution and partition between the mobile liquid phase and the stationary phase. The efficiency and speed of separation can be increased many folds. It has thus become a versatile separation technique, which has many uses both in clinical laboratory for estimation of a number of substances present in minute amounts in body fluids, as well as in the field of research and development. For quantitation of analytes it is a very sensitive and precise tool. Although the equipment is expensive, but it has advantages of being a very sensitive and precise method, at the same time the cost of analysis and maintenance is reasonable. Some of the applications are the identification, quantitation and analysis of haemoglobin variants, drugs, toxic substances, amino acids, carbohydrates, and metabolites of drugs and hormones. The solvents must be degassed to eliminate formation of bubbles. The pumps provide a steady high pressure with no pulsating, and can be programmed to vary the composition of the solvent during the course of the separation. This provides superior resolution and faster analysis. Detectors rely on a change in refractive index, UV-VIS absorption, or fluorescence after excitation with a suitable wavelength. There is a vast majority of column types, each for a specific type of mixture to be separated.

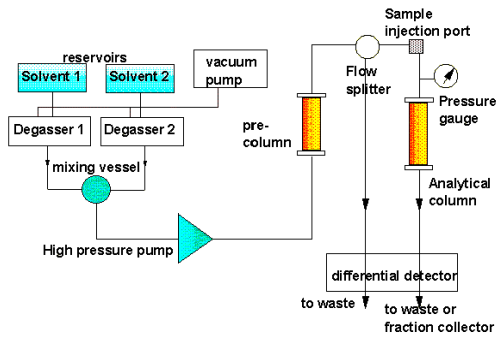


Figure 5.4: Schematic diagram of high performance liquid chromatography (HPLC)

GAS CHROMATOGRAPHY

Gas chromatography makes use of an inert gas (helium, argon or nitrogen) as mobile phase to carry the solute through the column. It is more suited for volatile organic compounds. It consists of a flowing mobile phase, an injection port, a separation column containing a stationary phase, and a detector (Figure 5.5). The organic compounds are separated due to differences in their partitioning behaviour between mobile gas phase and stationary phase in the column. The injection port is a rubber septum through which a

syringe needle is inserted to inject the sample. The injection port is maintained at a higher temperature than the boiling point of the least volatile component in the sample mixture. Since the partitioning behaviour is dependent on temperature, the separation column is usually contained in a thermostat-controlled oven. Separating components with a vide range of boiling points is accomplished by starting at a low oven temperature and increasing the temperature over time to elute the high boiling point components. Most columns contain a liquid stationary phase on a solid support. Separation of low molecular weight gases is accomplished with solid adsorbents. Commonly used detectors include thermal conductivity, flame ionisation, atomic emission, electron capture, photo ionisation, flame photometric, Chemiluminescence spectroscopy, and nitrogen phosphorous types.

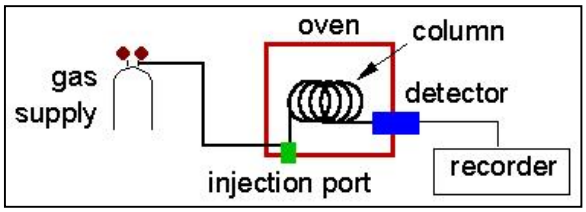


Figure 5.5: Schematic diagram of gas chromatography

There are three types of gas chromatography:

1. **Gas adsorption chromatography**: It involves a packed bed comprised of an adsorbent used as the stationary phase. Common adsorbents are zeolite, silica gel and activated alumina. This method is commonly used to separate mixtures of gases.
2. **Gas-liquid chromatography**: It is a more common type of analytical gas chromatography. In this type of column, an inert porous solid is coated with a viscous liquid, which acts as the stationary phase. Diatomaceous earth is the most common solid used. Solutes in the feed stream dissolve into the liquid phase and eventually vaporise. The separation is thus based on relative volatilities.
3. **Capillary gas chromatography**: It is the most common analytical method. Glass or fused silica comprises the capillary walls, which are coated with an absorbent or other solvent. Because of the small amount of stationary phase, the column can contain only a limited capacity. However, this method also yields rapid separation of mixtures.

All tissues/infective agents have genetic material in the form of DNA or RNA. The nucleotides are the building blocks of DNA/RNA and have certain species-specific sequences. Detection of specific DNA/RNA sequence of some organism/infective agent in a given sample is an evidence for its existence in the test material. Various methods like hybridisation techniques have been used for detection of target genetic material. These techniques always had a problem of sensitivity when number of target molecules in the sample was low. To overcome this problem, various amplification techniques like Polymerase Chain Reaction are used which amplify the small number of DNA/RNA molecules present in the sample and make its detection/quantitation possible. In addition to the DNA amplification techniques, highly sophisticated strategies like Branched Chain DNA (b DNA) signal amplification (which is based upon amplification of the signals and not DNA) are now in use for detection/quantitation of nucleic acids. The common amplification techniques are:

- Polymerase Chain Reaction (PCR)
- Ligase Chain Reaction (LCR)
- Nucleic Acid Based Amplification (NASBA)
- Strand Displacement Assays (SDA)

Polymerase Chain Reaction (PCR) is the most popular and most commonly used technique in pathology laboratories. It is used to amplify the specific region of DNA, in a reaction vial/well, in order to produce enough DNA to be adequately tested/detected. In case target is RNA, it is first converted into DNA with the help of Reverse Transcriptase (RT) enzyme and then subjected to amplification by PCR. In PCR, target DNA sequence is amplified with the help of primers, Deoxyribose Nucleotide Triphosphates (dNTPs) and polymerase enzyme. The primers are custom-made short DNA fragments and are usually 20-40 nucleotide long. The DNA sequence of the primer is complementary to the target sequence. The amplification is carried out in automated heating equipment called thermocycler, which has a program for cyclic heating at varying temperatures. Each cycle in a thermocycler results in duplication of the DNA molecules present before start of the cycle. In this way even a small number of DNA molecules is amplified. The amplified products are then detected by techniques like electrophoresis, ELISA and chemiluminescence. Various types of reagent kits for PCR tests are commercially available. Following are the basic steps for PCR

in a laboratory:

- DNA/RNA extraction from the sample
- Amplification
- Detection

To control the possible problem of contamination/carryover it is traditionally recommended that specimen extraction, amplification and detection should be carried out in different areas in a laboratory. RNA/DNA extraction from the sample is carried out manually according to the given protocol. Amplification takes place in thermocycler (Figure 5.6). Following steps take place at their particular temperatures provided in a cyclic manner through a thermocycler:

- **De-naturation** of the double stranded DNA at 94°C
- **Annealing** of the primers with the complementary DNA sequence in the target DNA at 54-60°C
- **Extension/lengthening** of the primer and formation of double stranded DNA molecule from single stranded at 72°C. The extension of primers takes place by the help of polymerase enzyme and Deoxyribose Nucleotide Triphosphates (DNTPs). The extension results in conversion of single stranded DNA into double stranded DNA and doubling of initial number of DNA molecules.
- The newly formed double stranded DNA is again denatured and converted into single stranded DNA and the whole process is repeated in the form of repetitive cycles. After 25-30 cycles, one molecule of the target DNA can be amplified to produce over 100 million DNA molecules.
- The process of amplification is followed by **detection/quantitation** of the amplified products. That may be manual or automated, depending upon the particular equipment and test protocol in use.

PCR technique and equipment has undergone remarkable improvements/modifications with passage of time and now has vast applications. The technique has proven its usefulness in different settings by its applications in infectious diseases, genetic disorders, antenatal diagnosis (page 301), oncogenes/oncological studies, anthropology and medicolegal science. PCR is being used for detection, genotyping and quantification of the disease-causing agents like hepatitis C virus, hepatitis B virus (page 206), cytomegalovirus and *mycobacterium tuberculosis*. The technique is also being exploited for prenatal diagnosis of disorders like β-thalassaemia, α-thalassaemia, sickle cell disorders, trisomy 21, trisomy 18, X chromosome aneuploides, haemophilia, deuschene muscular dystrophy, phenyleketonuria, cystic fibrosis, foetal sexing and foetal RhD typing. It is also helpful in disorders like Bcl2 gene rearrangement and can also be utilised for identification of a deceased person, or a criminal suspect.

Figure 5.6: Thermocycler: Perkin Elmer 9600

Figure 5.7: Real Time PCR - Cepheid Smart Cycler

A wide range of automated thermocyclers with better amplification and detection technologies are now available. Real Time PCR (Figure 5.7) offers automated facility for detection and quantitation of the target molecules during the process of amplification and no separate testing for detection of amplified products is required. As the name suggests, real time PCR is a technique used to monitor the progress of a PCR reaction in real time. Real Time PCR is based on the detection of the fluorescence produced by a reporter molecule, which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the

double-stranded DNA or sequence specific probes. In Real-time PCR, the one can quantify the minimal amounts of nucleic acid accurately in comparatively less time. The specimens are processed in the automated equipment after extraction and results are produced in a specified time in a computer format. Moreover, there is no need for the post PCR processing which saves the resources and the time. These advantages of the fluorescence based PCR technique have completely revolutionised the approach to PCR-based quantification of DNA and RNA. Real-time assays are now easy to perform, have high sensitivity, more specificity, and provide scope for automation. These assays are currently in use for qualitative as well as quantitative testing for various infective agents like HIV, hepatitis B virus, hepatitis C virus and Cytomegalovirus.

SPECTROSCOPY

Spectroscope is an instrument, which splits the visible light into its components. The areas of light absorption in the spectroscope are seen as vertical black lines called Fraunhofer lines. Wavelength of various spectral colours is given in Table 3.2. Spectroscopy is the procedure to observe the absorption spectrum of an analyte in liquid (biological pigment or abnormal substance). It is of two types:

a. Direct vision spectroscopy
b. Hartridge reversion spectroscopy

DIRECT VISION SPECTROSCOPY

Procedure

1. Place the eye to the eyepiece of spectroscope and view the sky through the instrument, but do not point towards direct sunlight.
2. Close the slit 'S' by turning the milled ring, then reopen the slit slightly until the spectrum is visible.
3. Adjust the eyepiece until the colours are focused and the Fraunhofer lines, which are due to absorption of light by different elements in the sun's atmosphere, can be clearly seen as fine vertical black lines across the spectrum. Fraunhofer lines are invisible unless a very narrow slit is used.
4. Check that the "D" line of the sun's spectrum, which occurs at 589 nm in the orange-yellow, corresponds with the position of the 589 reading on the scale.
5. Place the solution in a test-tube (for examining blood, a dilution >1:50 is used).
6. Position the sample tube in front of the slit and observe through the eyepiece. Record the position of any absorption bands seen in relation to the spectral colours and Fraunhofer lines. If possible compare with a solution of known composition.

HARTRIDGE REVERSION SPECTROSCOPY

Components

- Light source (Neon bulb)
- Tube container or cell
- Prism
- Filter
- Eyepiece

All these parts are mounted on a stand. When the neon light is switched on, light is split into two spectra, which are in contact but reversed. These two can be made co-linear with the movement of a screw. Similarly the absorption band in one spectrum can be made co-linear with the corresponding band. Spectroscopy assists in the identification of many pigments especially Hb and its derivatives. Following are the different pigments detected by this procedure:

- Hb in the serum
- Hb in the urine
- Methaemoglobin
- Sulphaemoglobin
- Carboxyhaemoglobin

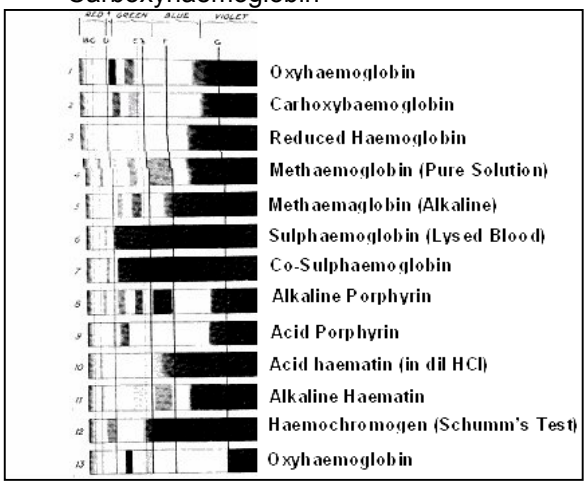


Figure 5.8: Adsorption spectra of haemoglobin and its derivatives

DETECTION OF CARBOXYHAEMOGLOBIN

Normal blood is diluted 1:300 in dilute ammonia solution (it prevents the precipitation of plasma proteins). It is placed in the cell of the spectroscope. The instrument is set in such a way that band of the spectra of oxyhaemoglobin overlap exactly. Now the patient's blood is diluted in the same way and placed in the spectroscopic cell in place of normal blood.

There should be no disturbance of the instrument adjustment for accuracy. If this test sample contains carboxyhaemoglobin there will be slight shifting of bands. They will no longer overlap each other. This shift is towards the violet colour of spectrum (Figure 3.5). This test will give a rough estimation of carboxy haemoglobin. It can detect 50% or more saturation with CO. This method becomes more sensitive if the test is done in a dark room or with a green filter. The patient's blood is then placed and the mean reading is noted. Even the slightest difference in the position of the absorption band should be noted. This method can determine 10-20% saturation of Hb with CO. If a blood sample is completely saturated with CO, the shift between the bands is 60° Angstrom. Saturation of sample with CO can be calculated according to this standard. (see also CARBON MONOXIDE on page 373)

SOLUTIONS

A **solution** is a homogeneous mixture of two or more substances. The component of the solution present in smaller amount or the one dissolved is called **solute** and the component in a greater quantity or in which the solute is dissolved is called **solvent**. For example in 10% glucose solution glucose is solute and water is solvent, while in 70% alcohol, water is solute and alcohol is solvent.

TYPES OF SOLUTIONS

Physical nature: On the basis of physical nature solutions are classified into three categories:

- Solids
- Liquids
- Gases

Nature of solution and Solvent: On the basis of the nature of solute and solvent there are nine possible forms of solutions as given below with examples:

a. Solid in solid: brass (copper and zinc)
b. Solid in liquid: salt in water
c. Solid in gas: smoke in air
d. Liquid in liquid: alcohol in water
e. Liquid in solid: Mercury in silver (amalgam)
f. Liquid in gas: steam
g. Gas in gas: air
h. Gas in solid: hydrogen in palladium
i. Gas in liquid: formalin

Concentration

1. Percent Solution: It contains the amount of solute as parts per 100 units of solution. The three categories of percent solution are:
   a. Weight by weight (W/W).
   b. Volume by volume (V/V)
   c. Weight by volume (W/V)

   For example a 5% sodium chloride solution contains 5g of sodium chloride in 100 ml of solution.
2. Molar Solution: Mole is defined as the gram molecular weight of a substance (molecular weight taken in gram). One mole of any substance will contain equal number of molecules given by the Avogadro's Number (6.024×10^{23}). Molarity is defined as the number of moles of the solute dissolved per litre of the solution. Molarity is expressed as moles per litre (mol/L) or milimoles per litre (mmol/L). One mole of any substance dissolved per litre of any solution will result in concentration of 1 mole (or 1M). A 1M solution of sodium chloride can be prepared by dissolving 58.5 g NaCl to a final volume of 1L. (molecular weight of NaCl is 23+35.5=58.5). Some commercially available chemicals may not be 100% pure, therefore, while preparing solutions of those substances their purity has to be taken into consideration. For making a molar solution of an acid following equation can be used:

$$V = \frac{M \times m \times 100}{T \times Sp\ Gr}$$

   Where:
   V = the required volume
   M = the molecular weight of the acid.
   m = required molarity
   T = percentage of acid
   Sp Gr = specific gravity

   For example if 0.02 molar solution of H_2SO_4 is to be prepared when provided H_2SO_4 has a percentage of 40 and specific gravity of 1.8: then

   Molecular weight (M) = 98
   Percentage (T) = 40
   Specific gravity (Sp Gr) = 1.8
   Required molarity (m) = 0.02 = m

$$V = \frac{98 \times 0.02 \times 100}{40 \times 1.8} = 2.72$$

   This means that 2.72 ml of given H_2SO_4 dissolved per litre of solution will make a dilution of 0.02 moles. Many salts contain water of crystallisation (hydrated salts). Their molecular weight can differ. This fact should be taken into account while preparing solutions of such salts. The molecular formula is usually given on the packing.
3. Normal Solution: Normal solution contains one gram equivalent of any substance per litre of solution. The normality is defined as

the number of gram equivalent weight per litre of a solution[1]. Equivalent weight of a substance is equal to the molecular weight of substance divided by the valence.

$$\text{Equivalent Weight} = \frac{\text{Molecular weight}}{\text{Valence}}$$

Calculation of equivalent weight

a. **Acid**: Equivalent weight of an acid is obtained by dividing the molecular weight of acid with the number of hydrogen ions. Sulphuric acid has molecular weight of 98. In solution it gives two H^+ ions. Therefore, equivalent weight will be 98/2=49 g.

b. **Bases**: Inorganic bases contain OH^- ions as their functional group. The equivalent weight of a base is obtained by dividing the molecular weight with the number of OH^- ions e.g., for sodium hydroxide molecular weight is 40. One OH^- ion is liberated in solution and thus its equivalent weight is also 40 g.

c. **Salts**: Equivalent weight of a salt is equal to the molecular weight divided by the valence number of metal ions present in the salt. Copper in copper sulphate has a valence of 2. Eq Wt of $CuSO_4$ is equal to its molecular weight divided by 2. But in sodium sulphate (Na_2SO_4) the valence of Na is 1 but two Na atoms are present. Therefore, the total valence of metal ion is 2. Thus the equivalent weight of Na_2SO_4 is equal to its molecular weight divided by 2.

4. Standard Solution. A solution of known concentration used for calibration is called a standard solution. These are commercially available or can be prepared in-house by dissolving exact quantity of a pure substance in its solvent or preservative solution..

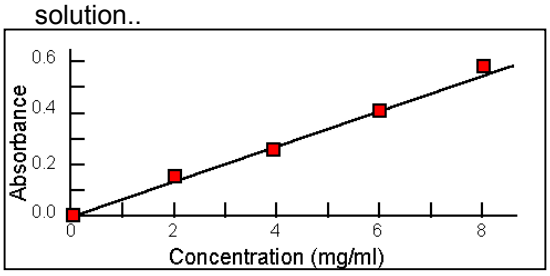


Figure 5.9: A standard, working or calibration curve

[1] By definition, normal solution contains one-gram equivalent weight of any substance per litre of solution. Thus 'Normal Saline' containing 9g/l NaCl is a misnomer because 9 g NaCl is approximately one sixth of the equivalent weight, and it should be called 'isotonic saline' or 'physiologic saline' being equal to plasma in tonicity.

PREPARATION OF CALIBRATION CURVE

A standard, working or calibration curve is a plot of the analytical signal (the instrument or detector response) as a function of analyte concentration. These curves are obtained by measuring the signal (absorbance) from a series of standards of known concentration (Figure 5.9). The standard curve can then be used to determine the concentration of an unknown sample or to calibrate the linearity of an analytical instrument. These are mostly used for colorimetric determinations (Preparation of calibration curve/chart on page 249). However, these are also required in RIA, ELISA and immunodiffusion. To illustrate the whole procedure, preparation of a calibration curve for blood glucose is described in some detail.

Requirements

Reagents

1. **Stock standard**: It is prepared by dissolving 360 mg pure, dried, analytical grade glucose powder in 100 ml saturated solution of sodium benzoate[2].
2. **Working Standards**: Prepare working standards by diluting stock standard as indicated in Table 5.3.

Graph Paper: Graph papers are of three kinds, linear-linear, log-linear and log-log. To plot absorbance against concentration of glucose in standard curve linear-linear graph paper is used.

Table 5.3: Preparation of working standard for a standard curve of glucose

TUBES →	1	2	3	4	5
Blood glucose (mg/dl)	0	90	180	270	360
Blood glucose (mmol/L)	0	05	10	15	20
Volume of Stock standard (ml)	0	0.25	0.5	0.75	1.0
Isotonic sodium chloride solution (ml)	1.0	0.75	0.5	0.25	0

Procedure

1. Set up 5 test tubes in a rack and proceed as shown in Table 5.3.
2. Process the whole batch of five tubes according to the method sheet.
3. Take the absorbance readings up to three decimal points and plot each absorbance reading against its corresponding concentration on a linear-linear graph paper.
4. Join all the points, which must be on or around a straight line. If the line starts deviating at high concentrations, determine the limit of linearity from the point of deviation. The relationship of absorbance and concentration can only hold good up to

[2] Sodium benzoate acts as a preservative for glucose. It is needed only if the glucose solution needs to be kept for sometimes. In case it is prepared and used as fresh, the use of this preservative can be omitted.

that limit of linearity.

5. From this curve a table can be prepared showing concentration of glucose against each absorbance unit.
6. Alternate to this table, one can calculate the factor for each analyte by dividing known concentration of standard by its absorbance as under (see equation on page 18):

   Factor (F) = C^S/A^S*

   The unknown concentration can then be obtained simply by multiplying this factor with the absorbance of unknown as:
   $C^U = FxA^U$*

Checking calibration curve: Some procedures require preparation of fresh calibration curve with each run of tests. However, in other cases it can be periodically checked by running controls. The calibration curve needs to be checked or made afresh whenever pipettes, reagents, standards, instruments, or technicians are changed.

5. Stock Solution: Sometimes a concentrated solution of a salt or chemical (Trichloracetic acid, TCA page 50) is prepared form which working solutions are made by dilutions. A dilute solution can be prepared from a stock solution by using the following formula:

   $C_1 \times V_1 = C_2 \times V_2$ (1)

Where

C_1 = concentration of stock solution
V_1 = volume of stock solution to be diluted
C_2 = final concentration
V_2 = final volume

To prepare 0.005 molar solution of NaCl from 100 ml of a 0.1 molar stock solution:

V_1 = 100 ml
C_1 = 0.1M
C_2 = 0.005M
V_2 = ?

$$V_2 = \frac{C_1 \times V_1}{C_2} = \frac{0.1 \times 100}{0.005} = 2000 \text{ ml}$$

Thus 100 ml from stock solution needs to be diluted to 2000 ml with distilled water to have a 0.005 molar solution.

BUFFERS

In many chemical reactions it is important to keep the *p*H constant. One needs to have methods for maintaining relatively constant pH. Solutions used for this purpose are called buffers. These are composed of a weak acid (or base) and its salt. Acetic acid and sodium acetate mixture in solution makes one buffer system. There are a number of buffer solutions commonly used in a laboratory. Their method of preparation is given in Appendix II: Preparation of common buffers on page 417.

*p*H INDICATORS

An indicator is the salt of a weak acid or base that exhibits one colour in the free unionised form and another colour in the ionised salt form. *p*H determines the relative amount of salt and acid (or base) form of an indicator, thus the colour. The colour changes with change in *p*H over a certain range. When used in titration it reflects the completion of the chemical reaction e.g., phenol red is yellow at *p*H 7.1 but turns to faint pink colour at *p*H 7.2. *p*H Indicators can also be used to have an estimate of *p*H of a solution or body fluid. Previously red and blue litmus papers were in use to determine the acidity or alkalinity. They had a broad range, thus have been largely replaced by indicators covering very narrow *p*H range. Modern laboratories use *p*H meters for measuring pH. These instruments are equipped with *p*H electrodes (page 24). Some indicators and their preparation is as follows:

1. **Methyl orange (0.1%)**: Dissolve one g of methyl orange in distilled water and make the volume up to 1L.
2. **Methyl red (0.1%)**: Dissolve 1g in 1L of 95% alcohol.
3. **Phenolphthalein (1%)**: Dissolve 5g of phenolphthalein in 500 ml of 50% alcohol. It should be neutralised (as it is acidic) with 0.01 M alkali till a faint pink colour appears and then the colour is removed by addition of 1-2 drops of 0.01M HCl.
4. **Potassium chromate (10%)**: Dissolve 25 g of potassium chromate in about 100 ml distilled water. Any chloride present is neutralised by adding and filtering 1-2 drops of silver nitrate solution. Volume is made up to 250 ml. The commonly used indicators with their range of colour change are given in Table 5.4.

Table 5.4: *p*H range of some common indicators

Indicator	pH range	Colour
Bromocresol purple	5.2-6.8	Yellow to purple
Bromophenol blue	3.0-4.6	Yellow to blue
Bromothymol blue	6.0-7.6	Yellow to blue
Cresol red	8.0-9.6	Yellow to blue
Litmus	4.5-8.3	Red to blue
Methyl orange	3.1-4.4	Red to yellow

*C^S= concentration of standard, A^S= Absorbance of standard, C^U=concentration of unknown, and A^U=Absorbance of unknown.

Methyl red	4.2-6.3	Red to yellow
Phenol red	6.8-8.7	Yellow to red

ANTICOAGULATION AND ANTICOAGULANTS

Anticoagulation is a process by which clotting of blood is prevented. Many methods are used for anticoagulation. These are:

1. **Dilution**: When a small amount of blood is added to a large amount of fluid reagent. This dilutes coagulation proteins so much that clotting is prevented. Best example in laboratory practice is collection of blood for blood culture. Here 5-10 ml blood is added to 50 ml culture medium. This 5-10 times dilution prevents blood from clotting.
2. **Defibrination**: Although in true sense it is not anti-coagulation. Since both serum and cellular components remain in liquid state and only fibrinogen is removed so it may be regarded as controlled anticoagulation. Various methods are used for this purpose. For small amounts, up to 10 ml, blood is put in a tube containing glass beads. Tube is tilted repeatedly and rotated for 20-30 min. In this way fibrinogen clots around glass beads and other components of blood remain in fluid state. For larger quantities of blood (50 ml or more) a conical flask is used. Mouth of flask is closed with a rubber cap with a hole in its centre. A long glass rod is taken and around lower half of it, pieces of capillary tube are attached with heat. Upper part is passed through the hole in the cap. Collected blood is put in the flask and rod is rotated for 20-30 Min. Fibrin clots around the capillaries. This anticoagulated blood is good for almost all tests except determination of platelet count and coagulation tests.

Use of Anticoagulants: Anticoagulants are substances, which are added to blood in order to prevent coagulation process. Many anticoagulants are used for this purpose. These may be divided into two groups.

1. **Chemical anticoagulants**: These are mainly calcium chelating agents. These remove calcium ions, which are essential for coagulation process. These include EDTA, Citrate, Oxalate, etc.
2. **Biological anticoagulants**: These are substances, which oppose the action of a specific protein in coagulation pathway. Best example is Heparin, which acts against factor Xa and thrombin.

Depending upon physical nature, anticoagulants can also be divided into two groups:

1. **Solid anticoagulants**: All chemical anticoagulants can be used in solid form and are used so when dilution of blood is not desired. In such samples concentration of reagents is not changed.
2. **Liquid anticoagulants**: Biological anticoagulants are liquid. Chemical anticoagulants are also used in liquid form where a predetermined change in concentration does not affect the test. These are used where plasma is required. Best example is trisodium citrate, which is used as liquid for coagulation tests or platelet count.

PREPARATION AND USE OF IMPORTANT ANTICOAGULANTS

Ethylenediaminetetra acetic acid (EDTA)

This anticoagulant is used widely for routine work in haematology. EDTA binds calcium ions and thus acts as a chelating agent. As a result calcium is not available for coagulation, and the blood does not clot. EDTA is used as an anticoagulant in the CP (CBC) bottles. EDTA is used as dipotassium salt in concentration 1.25-1.75 mg/ml of blood. Since the salt is usually not pure concentration should be kept near upper limit. Three ml of blood is required for complete blood picture, therefore, 5 mg of salt should be present in each bottle. More than 2 mg/ml EDTA may result in reduced PCV and should be avoided. Prepare a solution of EDTA by dissolving 2.5 g of dipotassium EDTA in 100 ml distilled water or 1% formalin solution. Pipette 0.3 ml of this solution into each bottle and evaporate to dryness in a hot air oven at 60°C for 2 h or at 37°C for 24 hours. It is important that the EDTA in the CP bottle should be optimum. Excess of EDTA will result in swelling of platelets, which eventually break up resulting in false low count. Relative excess of EDTA can also occur if less amount of blood is added to the bottle, which reduces PCV. Violent shaking of the CP bottle in the air will result in formation of micro clots, which interfere in subsequent test. The bottle should be rubbed against a smooth surface in a to and fro motion or gently rotated between palms. It is not advisable to do coagulation studies on the blood, which has been anticoagulated with EDTA.

Trisodium Citrate

Trisodium citrate is the anticoagulant, which is used for coagulation studies and for ESR measurement. The mechanism of action is similar, to that of EDTA that is it binds with calcium ions and prevent coagulation. Trisodium citrate is used as 0.106 molar solution. This is

prepared by dissolving either 31.3g $Na_3C_6H_5O_7$ or 38g of $Na_3C_6H_5O_7$ $11H_2O$ per litre of distilled water. Nine parts of blood are added to 1 part of this anticoagulant so that dilution of blood is exactly 9/10. Excess of trisodium citrate will result in prolongation of the coagulation times while doing PT, PTTK and TT. Therefore it is essential that the amount of trisodium citrate should be exact. Lesser volumes of the anticoagulant will result in shortening of the coagulation times.

Heparin

Heparin may be used instead of the other anticoagulants. However, it should not be used to make blood films, because it gives a bluish discoloration to the background. It is ideal for osmotic fragility test. Heparin is used in a concentration of 15-20 IU per ml of blood. For chemical tests lithium heparin is used in concentration of 2 mg/10 ml of blood. To prepare 10 ml sample tubes prepare solution of 2 g lithium heparin in 100 ml distilled water. Distribute 0.1 ml of it in each sample tube. Stopper the tube and rotate, so that the fluid forms a layer on the sides of the tube in the lower half. Remove stopper and dry in the oven at 60°C for 2 hours. It is important to prepare a thin film on the walls of a sample tube because heparin otherwise is not quickly soluble. For certain tests, such as in tissue typing, sodium heparin in powder form is used.

Oxalate

Oxalate inhibits coagulation of blood by precipitation of calcium. Potassium oxalate is most commonly used and the concentration required is 2-3 mg/ml of blood. It can be used in finely powdered form. Add 9 mg of salt in each sample bottle for 3 ml of blood.

Sodium fluoride

It is most commonly used as a preservative in sample bottles for blood glucose. In larger amounts it acts as an anticoagulant but is not suitable in that concentration. In sample bottles for blood glucose it is used with potassium oxalate (2:3 ratio) or with EDTA (2:1 ratio). To prepare bottles, weigh 6 g sodium fluoride and 3 g disodium EDTA. Dissolve in 100 ml distilled water. Distribute 0.1 ml of it in each sample bottle and evaporate to dryness in a hot air oven at 60°C for 2 hours. These bottles are suitable for 3 ml blood.

ANTICOAGULANTS IN BLOOD BANKING

Collection of blood for transfusion requires to be anticoagulated and provided with an artificial energy source so that blood cells, particularly RBCs remain viable during storage. Many anticoagulants are available. Most important are ACD (acid citrate dextrose and CPDA (citrate phosphate dextrose with adenine. These are used in dilution of 1/10 (50 ml in a 500 ml blood bag).

PROTEIN FREE FILTRATES

For determination of some blood constituents it is necessary to remove plasma or serum proteins e.g., in case of lipaemic, icteric or haemolysed samples. A number of methods have been used for the preparation of protein free filtrate. In these methods a substance is added to combine with and precipitate the proteins, leaving desired constituents in solution. Most commonly used precipitants are tungstic acid, trichloracetic acid and zinc hydroxide. For preparation of filtrate, blood or serum is diluted in a definite ratio usually 1:10. After the protein is precipitated the solution is filtered or centrifuged and filtrate or supernatant is used for analysis. Followings are the commonly used methods for preparation of protein free filtrate.

Folin-Wu filtrate: It is the oldest method of blood deprotienisation.

Reagents

1. Sodium tungstate 0.30 mol/L. Dissolve 50 g reagent grade sodium tungstate in water to make 500 ml.
2. Sulphuric acid 0.33 mol/L. Dilute 1 volume of concentrated acid with 52.5 volumes of water, adding acid to water.

Procedure

For whole blood dilute 1 volume of blood with 7 volumes of water, then add 1 volume of sodium tungstate, mix and add 1 volume of 0.33 mol/L sulphuric acid solution and shake. For plasma or serum 1 volume of serum or plasma is mixed with 8 volumes of water and 0.5 volumes each of sodium tungstate and sulphuric acid and centrifuged after 10 min.

Trichloracetic acid filtrate: This may be used for the determinations of inorganic phosphorous and for other procedures requiring an acid filtrate. For a 1:10 dilution, one may use 1 ml serum or plasma and 9 ml 0.3 mol/L trichloracetic acid, allow it to stand for few min and then centrifuge or filter. Trichloracetic acid is a hygroscopic compound. It is supplied in crystalline form. Once the bottle is opened whole of it has to be dissolved in appropriate amount of water to prepare a stock solution (page 46). Working solutions of appropriate dilutions can then be prepared from this stock solution.

Somogyi filtrate: It is not commonly used now a

days.

Reagents

1. Zinc sulphate solution, 0.175 mol/L. Dissolve 50g of reagent grade zinc sulphate in water and dilute to 1L.
2. Barium hydroxide, 0.15 mol/L. Dissolve 47g of barium hydroxide in freshly distilled water and dilute to 1L.

Procedure

1 Add 1 volume of blood to 5 volumes of water.
2 Add 2 volumes of barium hydroxide and mix.
3 Add 2 volumes of zinc sulphate solution and mix. Centrifuge or filter. This produces a 1:10 dilution of the blood.
4 The same proportions and reagents are used for serum.

6. COMPUTER AND AUTOMATION IN THE LABORATORY

COMPUTER

Computer is an electronic device that accepts data as input, processes that data and produces results as useful information at a very high speed. Data is input or information entered to a computer system for processing. Output is the presentation of results of processing e.g., to a display, monitor, or a printed report or document. Some important definitions and terms used in relation to computers in general are given below:

Information Technology: IT in terms of computer is defined as the integrating of computing technology and information processing.

Internet: It connects and brings together tens of thousands of networks, millions of computers, and many more millions of users in every country. In short we can define Internet as "the network of networks".

Local Area Network (LAN): A LAN connects workstations in an office or a building. In most LANs one central computer is called as server. This performs a variety of functions for the other computers on the LAN called client computers.

Modem: Modem permits communication with remote computers via a telephone-line link. The fax modem performs the same function as a regular modem plus it has an added capability. It enables a computer to become a fax machine.

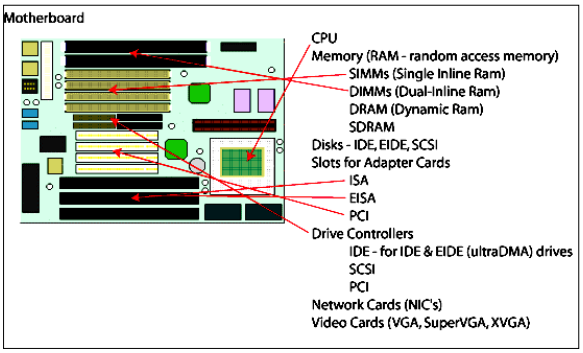
Bit: A bit (short for binary digit) is the smallest unit of data in a computer. A bit has a single binary value either 0 or 1 (off or on).

Byte: A byte is a unit of data that is equal to eight binary digits. A byte is the unit most computers used to represent a single character of a letter, a symbol or a typographic symbol (e.g., "g", "5", "?"). In this system the letter 'A' is represented by a byte consisting of a combination of 0s and 1s i.e., '01000001' and letter 'B' by '01000010' and so on.

Port: An access point in a computer system that permits communication between computer and mouse, keyboard & printers.

Power Supply: This component transforms alternating current (AC) into the direct current (DC) needed for the computer's operation. It also steps the voltage down to the low voltage required by the motherboard. Standard AC power from a wall outlet is converted to low voltage (2 to 12 Volt) DC power that can be used by the computer. Two hundred watts is the power an average computer requires.

Motherboard: It is the physical foundation of a computer on which processor and memory chips such as ROM and RAM are attached. It has multiple slots and connectors for linking other peripheral devices to the motherboard (main board, system board).



Chip: The integrated circuits or chips are tiny silicon chips into which thousands of electronic components are etched. The processor is also a chip.

Processor: It is an electronic device that can interpret and execute programmed commands for input, output computations and logical operations.

BIOS: BIOS stand for Basic Input Output System. The BIOS is a small chip on the motherboard that has the programmed instructions for start-up and self-testing of the computer. It consists off a small amount of memory to remember these instructions, setting the new **Plug and Play** devices, and also for handling the input and output of the data. The BIOS can be changed and updated.

ROM (Read Only Memory): It is a special type of internal memory, which cannot be altered by the user. On turning the computer on, a programme in ROM automatically readies the computer system for use and produces the initial display screen prompt.

RAM (Random Access Memory): It is a read and write memory, which enables data to be read and written to memory. All programmes

and data must be transferred to RAM from an input device or from primary storage device before programmes can be executed and data can be transferred. This memory area is the one in which all programmes and data must reside before programmes can be executed or data can be manipulated, interpreted.

Data: Data is just raw facts. Information is data that have been collected and processed into a meaningful form.

Database: Database is a collection of related data or pieces of information put together in an organised manner designed to meet the needs of various departments in an organisation.

Computer Virus: A computer virus is a programme (a block of executable code), which attaches itself to, overwrites or otherwise replaces another programme in order to reproduce itself without the knowledge of the user. A virus is a piece of computer software designed with bad intention and written to adversely affect one's computer by altering the way it works without one's knowledge or permission. Computer virus like biological virus needs a host to infect; in the case of computer viruses this host is an innocent programme. If such a programme is transferred to another computer, programmes on that computer will also become infected.

Hard copy: The output from a computer is in two basic forms, soft copy and hard copy. In hard copy one can get the physical copy in the form of printed report from a printer.

Soft copy: It is a temporary output that can be interpreted visually, as on a monitor or screen, where one can only see the result of the processing.

Hard Disk Drive (HDD): Hard disk is permanently installed, high capacity disk for permanent storage of data and programmes.

Computer Network: It consists of more than one computers linked electronically through a cable or telephone line to share resources and information. Computers in the same building, in the same city, or across the country can be connected.

Server Computer: It is a computer from a PC to a Super computer, which performs a variety of functions for its client computers, including the storage of data and application software. It acts as a central unit for a network.

Workstation: A high-performance single-user computer system with sophisticated input/output devices connected through cable with other workstations or computers is a workstation.

A typical computer consists of three main components: *CPU; Output devices; and Input devices.*

1. **CPU (central processing unit)**
   It has two fundamental sections, the control unit and arithmetic and logic unit. These units work together with random access memory (RAM). Control unit has three primary functions:
   - To read and interpret programme instructions.
   - To direct the operation of internal processor components.
   - To control the flow of programmes and data in and out of RAM.

   The Arithmetic and logical unit perform all computations (addition, subtraction, multiplication, and division) and all logic operations (comparisons).

2. **Output devices**
   These consist of monitor (screen) and printer. A monitor displays soft copy (temporary) output. A printer produces hard copy (printed) output. A set of speakers is for audio output. Printer is one of the most commonly used output device. There are several types of printers:
   a. **Dot Matrix Printer**: The dot matrix printer uses print heads containing from 9 to 24 pins. These pins produce pattern of dots on the paper to form the individual characters. The pins strike the ribbon individually as the print head moves across the entire print line in both directions. Dot matrix printers are inexpensive and typically print at speeds of 100-600 characters per second.
   b. **Ink Jet Printers**: The ink jet printers work in the same fashion as dot matrix in that they form images or characters with little dots. However, tiny droplets of ink form the dots. Ink jet printers form characters on paper by spraying ink from tiny nozzles through an electrical field that arranges the charged ink particles into characters at the rate of approximately 250 characters per second. The ink is absorbed into the paper and dries instantly. Various colours of ink can also be used.
   c. **Laser Printer**: Laser printer produces images on paper by directing a laser beam at a mirror that bounces the beam onto a drum. The drum has a special coating on it to which toner (an ink powder) sticks. Using patterns of small dots of laser beams, it conveys information from computer to a

positively charged drum to become neutralised, the toner detaches. As the paper rolls/passes the drum the toner is transferred to the paper, printing the letters or other graphics on the paper. A hot roller binds the toner to the paper.

3 **Input devices**
A pointing device for input is usually a mouse. A keyboard is for entering data by typing. A scanner, digital camera, or a microphone also act as input devices.

Data storage
One or more (physical or logical) permanently installed high capacity hard-disk drive(s) are provided for permanent storage of data and programmes. A floppy disk drive is used as an interchangeable diskette. A CD-ROM is an interchangeable storage device of very high capacity. Besides these there are other storage devices as well.

- **Zip Drive**: It is a storage device that uses optical technology together with magnetic technology to read and write to an interchangeable 100-1000 MB capacity disc.
- **USB Bar (Flash drive)**: USB stands for universal serial bus. This is a data storage device, also known as flash drive. Unlike other memory devices, it is in the form of a chip resembling the RAM fitted on the motherboard. This has the advantage of safe storage of data. The chances of accidental data loss, or data loss due to damage to the surface are minimum. The bar comes in storage capacity of 32-516 MB. It needs to be plugged to USB port, available on all modern PCs. If not available on any computer, one can be fitted on motherboard like any other hardware component. Once it is plugged in, its drivers have to be installed. Windows2000 can do it automatically, but prior versions of windows would require manual installation from the installation disc that comes with USB. The bar can also be used for safe data transfer from one PC to the other. It is very handy, so can be carried in pocket.
- **Tape drive**: A tape back up drive does not provide the random access required for everyday storage operations. These are only used as inexpensive back ups of large hard disc drives for security purposes.
- **DVDs**: It stands for digital video (or versatile) disc. Data in the form of video, audio, text or programmes is represented as on a CD-Rom, but the data storage capacity is much more (about 13 times) than a CD. The data is more compact and is stored in more than one layer. Like CD it also uses a laser beam to read the 'lands' and 'pits'. DVD drives can also read CDs.

Computer system consists of two main components, *hardware* and *software*.

1 **Hardware**: These are the hard components used in a computer such as motherboard, monitor, keyboard, mouse, various cards etc. Hardware is composed of physical parts and components of a computer such as: central processing unit (CPU) and main board (motherboard). Basic Input Output System (BIOS) or Read Only Memory (ROM) is contained in small integrated circuits on the board called chips. It also has many slots and connectors for communication ports, data storage devices such as floppy (FDD), hard (HDD), compact (CD) and video (DVD) disc drives; Random Access Memory (RAM) and Input/Output (I/O) devices.

2 **Software**: Software consists of a series of instructions written in a particular language understood by the computer, also called a computer programme. When a computer is given a command to perform any task it follows these pre-written instructions. Programmes are written for various tasks to be performed by a computer. Software are of various types:

- System Software: All the software used to operate and maintain computer system is called system software. The example of system software is *Operating System* (OS) or *Disc Operating System (DOS)*.
- Programme: Computer instructions structured and ordered in a manner that their execution causes a computer to perform a particular function are a programme. Programming is the act of producing such instructions or programmes (also called software). MS office is a programme (or application) written for office management. Similarly, LIMS (laboratory information management system) is a software (programme or application) specifically written for medical laboratory.
- Application Software: A collection of various programmes designed to carry out specific task by a computer to satisfy a user's specific needs are called Application Software. LIMS is application software.

USE OF COMPUTER IN MEDICAL LABORATORY

Computers are extensively being used in the field of medical laboratories. These have become an essential part of any laboratory. It is, therefore, mandatory that every laboratory

worker acquires the basic working knowledge about computers and to learn its proper use. The advantages of use of computers in laboratory are given in subsequent section on automation.

Computers are essentially utilised as components of laboratory equipment. Major contribution of computers is their use as an essential component of automated and semiautomated laboratory equipment. This has allowed automation of most of the laboratory procedures such as pipetting, mixing and centrifugation, incubation, photometry and integration/calculation of final result. One can feed a blood sample at one end of a large analyser and obtain results of as many as thirty or more different tests from the other end. Computers control all of these processes. Now a days there is hardly any piece of laboratory equipment, small or large, which does not incorporate computer in any form. Semiautomated equipment such as MICROLAB, SYSMEX or equivalents are also called **microprocessor-controlled** equipment as the computer component is only a microchip and is not obvious. On the other hand, large automated equipment such as SELECTRA or LIAMAT etc., have a visible computer component.

LABORATORY INFORMATION MANAGEMENT SYSTEM (LIMS)

It is used in laboratory to replace the old manual system of patient record keeping and report preparation. It performs the following tasks:

1. Registration of patients' personal or demographic data and allocation of a universal patient identification code number (Patient ID No).
2. Ordering tests to be performed on that patient and preparation of a receipt showing delivery date and time for each test.
3. Generation of a number of appropriate worklists for various departments.
4. Provisions for entry of result data for various tests once the tests are completed.
5. Preparation and printing comprehensive and complete test result report for a particular patient or department.
6. Maintaining various types of accounts.
7. Preparation of bills for patients of various organisations.
8. Preparation of various periodic (daily/monthly/quarterly/yearly) reports and returns of workload.
9. Retrieval of stored data in any form required at any time.
10. Research oriented data analysis.

Use of computers has greatly facilitated the working of laboratories. It has reduced the clerical mistakes, which were liable to occur at all stages. It has now become easy to feed all the relevant information into the computer. The information is automatically stored and is readily available for internal audit, research and planning. This saves wastage of paper, storage space and manpower.

INTERFACING OF AUTOANALYSERS WITH THE LABORATORY INFORMATION SYSTEM

For better laboratory management, improvement in performance, to reduce errors and the turn around time, the modern concept is to fully integrate these machines with the laboratory information system. In order to forego manual preparation of request forms and labels, bar code technology may be used. For efficient and timely sample transportation conveyer belts or pneumatic tube systems may be used. Automatic samplers are optional with many modern machines so that the technician may walk away from the machine and do other useful work. Some of the newer machines do automatic quality control and online support for quality control is available in real-time from the manufacturer by Internet.

AUTOMATION

Recent developments in electronic, robotics, computer technology and new analytical methods have been integrated to produce so-called automated laboratory analysers. This generation of equipment has greatly facilitated the work in busy clinical laboratories. Such equipment is usually expensive and requires expert engineers to maintain but has several advantages. Some of these are:

1. Manipulation of heavy workload with less manpower.
2. Reduction in time in completing the test.
3. Reduced consumption of reagents and microanalyses.
4. Precision and accuracy of results.
5. Integration of quality assurance into the test system.
6. Automatic printing of results thus eliminating clerical errors.
7. Distant communication of results.
8. Data storage and statistical analyses.

GUIDELINES FOR CHOOSING AN INSTRUMENT

The laboratory should define its budget and

scope of daily work etc. It can choose instrument from amongst the market. Factors to be considered in making a choice include capital expenditure, running and maintenance costs, ready availability for reagents/accessories/spare parts, size of instrument, requirement of services (water, compressed air, drainage, electrical supply with a stable voltage), reagents availability, storage and back up services etc. A committee should consider whether to buy or lease the instrument. Alternatively, the machine may be used on a reagent rental basis.

There is hardly a branch/department of Pathology where automation does not exist. Some examples of common automated equipment are as follows:

AUTOMATION IN HAEMATOLOGY

Several tests performed in haematology laboratory have been automated. Most important of these are blood counts, coagulation and blood grouping/cross matching.

AUTOMATION OF BLOOD COUNTS

Complete Blood Counts (CBC) form the main bulk of laboratory tests requested. By manual method it is difficult to do all of these with acceptable accuracy and precision. This was realised very early. In 1956 Wallace Coulter first described an electronic cell counter, which has revolutionised the haematology laboratory. Since then tremendous technological improvements have occurred in electronic blood cell counting and sizing. The market is now flooded by myriad of such instruments. The manufacturers have tall claims for these, which have to be verified before making a decision for purchase. Haematology analysers are now available for the needs of laboratory of any size. The range varies from simple blood cell counts and red cell indices to partial or full differential count, histograms of cell sizes and reticulocyte count. It is important, particularly in our country, to ensure that proper after-sale services and spares are available with the supplier.

TYPES OF AUTOMATED CELL COUNTERS

Fully automated instruments
In these only an appropriate blood sample is presented to the instrument. Some are capable of aspirating the sample themselves from containers placed on a turntable or similar device.

Semiautomated instruments
These require some steps, e.g., dilution, to be performed by the operator. They often measure a small number of components. These are mostly obsolete now.

PRINCIPLES OF AUTOMATED BLOOD COUNTING

1. **Measurement of haemoglobin concentration**
   Most automated counters measure haemoglobin by a modification of the manual cyanomethaemoglobin method. Due to high throughput of the instruments, measurements of absorbance are made at a set time interval after mixing of the blood and the active reagents but before the reaction is completed. In order to achieve this the standard HiCN technique is modified with respect to *p*H of reaction, temperature and concentration of the reagents. Usually a non-ionic detergent is used to ensure rapid cell lysis and to reduce turbidity. Alternatively, in some instruments sodium lauryl sulphate is used to measure haemoglobin. This is due to the fact that cyanide used in HiCN method is a highly toxic substance.

2. **Particle (Cell) Counting**
   The two basic types of technologies used for blood cell counting are aperture (electrical) impedance counting and optical method (light scattering) counting. In these methods a large number of cells are counted rapidly. This leads to a high level of precision and reproducibility, which sharply contrasts with the results obtained for blood cell counting by manual techniques. These technologies have made RBC count, MCV and MCH of much greater clinical relevance.

   a. **Aperture impedance counting**
      Blood cells do not allow electrical current to pass through them, i.e. they impede the passage of electrical current. There are certain diluents, which allow electrical current to pass through them. This difference forms the basis of cell detection by this technology. The cells are highly diluted in a buffered electrolyte solution. This fluid passes through a small aperture. A constant current passes through two electrodes on either side of it. As a blood cell passes through it, electrical conductance in the aperture is decreased. This generates an electrical impulse, which is proportional to the size of the blood cell. These impulses are

sorted electronically and split to count WBC, RBC and platelets.

b. Optical method (light scattering) counters

The blood cells scatter light to a variable extent and at various angles, depending upon their size, shape, nuclear lobes, presence of granules, etc. This forms the basis for blood cell detection and counting by electro-optical methods. The blood cells are suitably diluted. The diluted blood cell suspension is made to flow through an aperture in a way that the cells pass in a single file in front of a light source. The light is scattered by the cells. This scatter is measured by photo-multiplier tube (PMT) or photodiode, which converts it into electrical impulse. These impulses are then sorted to count WBC, RBC, Platelets and three part differential (neutrophils, lymphocytes and un-identified cells)

3. Automated WBC differentials

Some automated blood counters have a WBC differential counting capability and provide three/five/seven part WBC differential counts. Abnormal cell populations may be flagged to be confirmed by microscopy. Three part differential counts are based on different volume of various cell types. In optical detection methodology this may be augmented using flowcytometry. In electrical impedance methodology, cells are further characterised with radio frequency current or low and high frequency electromagnetic current. Some counters use cytochemical stains to differentiate between various WBC.

4. Platelet counting

Platelets can be counted in whole blood using same techniques as employed for red blood cells. Usually platelets are counted in the same channel as used for red blood cell detection with a threshold set to separate red blood cells from platelets.

5. Reticulocyte counts

Reticulocytes contain RNA. There are fluorescent as well as traditional dyes, which combine with RNA, and reticulocytes can thus be counted.

Graphical representation of data

These instruments also produce a graphical representation of the data in the form of histograms or scatter plots. These may either be in colour or in black and white. These graphs provide further valuable information. These show patterns which correlate well with various abnormalities in the blood film. This alerts to the possibility of an abnormality, which can then be confirmed by examination of a blood film.

CALIBRATION OF HAEMATOLOGY AUTOANALYSERS

These machines are calibrated in the factory. However, calibrators are available which can be used to calibrate them when required. These calibrators are quite expensive. The manufacturer supplies details for calibration. Alternatively, these may be calibrated by using single channel semi automatic analyser for RBC count, WBC count and platelet count. The haemoglobin is calibrated using cyanmethaemoglobin method, while the PCV is calibrated using the micro-haematocrit method.

EXAMPLES OF HAEMATOLOGY AUTOANALYSERS

The major manufacturers include Beckman Coulter, Sysmex, Technicon-Bayer, Cell Dyn series of Abbott Diagnostics, Cobas of Roche Diagnostic systems. Various models are available by each manufacturer.

PRACTICAL IMPLICATIONS OF HAEMATOLOGY AUTOANALYSERS

These instruments, to be useful need proper maintenance and backup services. The laboratory should ensure proper internal quality control as well as external quality assessment of these machines. Various instruments use technologies like hydrodynamic focusing or sheath flow, electronic editing, sweep flow etc. These machines are usually closed system, with reagents, controls, calibrators all being supplied by the manufacturer himself.

AUTOMATION IN HAEMOSTASIS

Automated coagulation analysers

A number of automated and semi automated coagulation analysers are available. The choice of an analyser depends on the workload, repertoire and cost implications. A thorough evaluation of the current range of analysers is recommended before purchase.

Most equipment is based on clotting assays. Formation of fibrin clot results in change in optical density of the reaction mixture. The end point is determined by decrease in absorbance of light due to formation of clot. If coagulation analysers are used it is important to ensure that the temperature control and the mechanism for detecting the end point are functioning properly. Although such instruments reduce observer error when a large number of samples are tested, it is important to apply stringent quality control at all times to ensure accuracy and precision.

Automation in Platelet Function Tests

An *in-vitro* system for measuring platelet vWF function PFA-100 (Dade Behring) is now available. The instrument aspirates a blood sample under constant vacuum from the sample reservoir through a capillary and a microscopic aperture cut into a membrane. The membrane is coated with collagen and either adrenaline or adenosine 5' diphosphate. It, therefore, attempts to reproduce under high shear rates vWF binding, platelet attachment, activation and aggregation, which slowly builds a stable platelet plug at the aperture. The time required to obtain full occlusion of the aperture is reported as the **closure time**. Collagen/adrenaline is the primary screening cartridge and the collagen/ADP is used to identify possible aspirin use. The PFA-100 system may reflect vWF platelet function better than the bleeding time but it is not sensitive to vascular collagen disorders.

AUTOMATION IN BLOOD BANKING

The increase in workload and the requirement of reliability of test results has resulted in introduction of automation for various serological procedures in blood bank. These include blood grouping, antibody screening, anti-RhD quantitation and screening of blood for transmissible diseases. Various equipments used for this purpose are designed for large workload and are not suitable for an ordinary hospital blood bank.

Most of the automated systems used in the blood bank are based on following techniques:

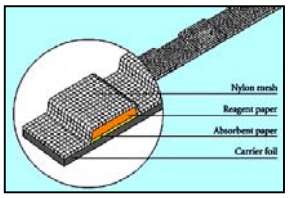
1. **Individual reaction wells**: In this anti-sera and red cell suspensions are automatically poured in individual reaction cells on a tray. This is then centrifuged and reactions are read by change in absorbance of light passed through the bottom of the cell. Example of this equipment is Kontron Groupamatic System.
2. **Microplate Procedure**: In this system serological reactions are carried out in microplates. The underlying principle is the same.
3. **Continuous Flow System**: In this system antiserum is allowed to react with red cell suspension in a continuous system of coils. Technicon Autogrouper utilises this system. It is interfaced with computer for recording of results.
4. **Gel Microcolumns**: In this system antisera and red cell suspension are allowed to act in solid phase sephadex columns. Special centrifuge is required for cards holding a number of columns. This technique has the advantage of better reproducibility and avoidance of washing step. Example is DiaMed and DiaGel Systems.

AUTOMATION IN MICROBIOLOGY

Like other departments automation has also been introduced in the Microbiology laboratory. The range of its application varies from automated pouring of culture plates to detection of bacterial growth, identification through chemical reactions and performance of antibiotic sensitivity. Automation in these areas not only reduced the time for reporting results but a greater degree of precision and accuracy has been achieved in performing various tests.

AUTOMATED URINE STRIP READER

This instrument (Clinitek-100) is a semiautomated, bench-top, dry chemistry urine analyser designed to read reagent strips for urinalysis. The instrument is initially configured for Multistix[10] (10 parameters), But Multistix[9] (9 parameters), and Uristix (2 parameters) can also be used. The reagent strips contain areas for testing glucose, bilirubin, ketones, specific gravity, occult blood, pH, protein, urobilinogen, nitrite and leukocytes. The instrument works on the principle of reflectance. It analyses the colour and intensity of the light reflected from the reagent area and displays the result in clinically meaningful units. No calculations are required. It saves time and labour. The strips provide rapid test results and



are often less expensive than performing same tests by wet chemistry. The reliability of reagent strip test results depends on the correct urine sampling, storage, use, control of the strips and knowledge of the causes of false positive and false negative reactions.

AUTOMATED PLATE POURING UNIT

The automated plate-pouring unit is used to dispense a prefixed amount of sterile medium into petri dishes. In modern era of medical microbiology, increasing workload in a reference laboratory warrants a system of media preparation that is capable of rapidly dispensing large quantity of sterile media. In this equipment, each petri dish is taken from a carousel (capacity of 216 plates) and transported by a studded belt between guide rails to the central position where medium is dispensed. During this movement one guide rail tilts the dish cover sufficiently to allow the media nozzle from the peristaltic pump to pass between the lid and the base. After the dish is filled with the preset volume of media, the petri dish is transported to the stacking unit. During this stage the petri dish lid is returned to the base. Whilst the lid is raised the petri dish is enclosed within an enclosed space protected with UV light. The stacking station will stack the petri dishes in a column and the completed stack is pushed onto the stacking rail that can hold up to 6 columns. Complete filling of rail is indicated by an LED (Figure 6.1). The whole process is completed by non-touch technique, reducing the chances of contamination. There is saving of time and labour.

Figure 6.1: Automated plate-pouring unit

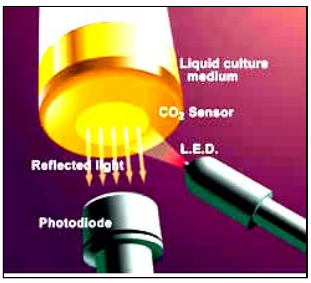
BACTEC RADIOMETRIC SYSTEM

This is a rapid culture system in which growth of Mycobacteria can be obtained in 7-12 days and a further 5-7 days are required for antibiotic sensitivity. A complete report of Mycobacterial culture and sensitivity can be obtained in 2-3 weeks instead of 8-12 weeks taken by traditional method.

Principle: Radioactive carbon (^{14}C) as part of palmitic acid is incorporated in the medium. Mycobacteria if present, grow, utilise ^{14}C and produce CO_2 containing radioactive carbon. This radioactivity detected by the instrument is directly proportional to the growth of mycobacteria and is displayed in the form of growth index. An index of 100 or more is considered positive. Four ml quantity of Bactec 12B medium is specifically used for mycobacterial culture in this system based on Middlebrook 7Ha liquid medium.

BACT ALERT

This is a rapid bacterial/fungal culture system for blood or sterile body fluids in which the growth of bacteria can be detected within 1 hour to 7 days (Figure 6.2). If growth is displayed as positive, then it is sub-cultured on other culture media. If the test is negative after 7 days it indicates no growth (the time period is adjustable).



Principle: It is same as for the Bactec except that the ^{14}C labelled CO_2 is detected non-radiometrically by a colorimetric signal generated by an exciter wavelength. Bact Alert aerobic and anaerobic medium is used in this system. Specimens are sub-cultured on days 1, 2, 4 and 7, however, this system does not require sub-culturing as a routine, thus saving time and effort.

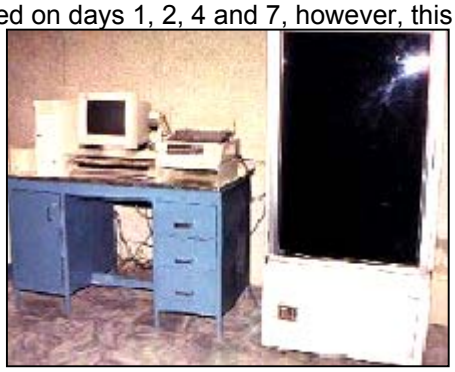


Figure 6.2: Bact Alert

AUTOMATION IN CHEMICAL PATHOLOGY

Chemical pathology laboratory techniques include sample preparation, pipetting of precise

volumes, mixing, incubation, dialysis, separation and photometry etc. These have also been benefited by the global advancements in technology, as the automation has been introduced in this field gradually over last four decades. This automation has evolved through many stages. It started with the invention of single channel Autoanalyser (AAI), developed by Technicon® in late fifties. The system had separate components or modules such as sampler unit, proportionate (peristaltic) pump, mixer coils, dialyser, oil bath, and photometer with recorder, all linked together with Teflon or glass tubes. Samples used to be introduced into the system in a sequence, separated by air bubbles. Later on same company developed sequential dual and subsequently, multichannel analysers variously known as AAII, SMA6, SMA12, SMAII, SMAC etc. At the same time other manufacturers also entered the field and a magnitude of analysers came into being. Some important members are COBAS, Dupont ACA, Abbott Laboratories TDX and IMX, Coulters Kem-O-Lab, Hycel-M, Beckman ASTRA, Merck's MICROLAB and SELECTRA, LIAMAT, METROLAB, IMMULITE, and many others.

TYPES OF CHEMICAL ANALYSERS

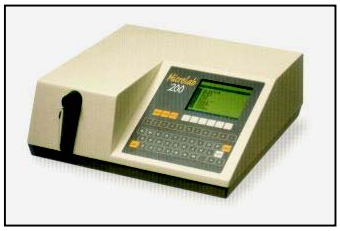
There are three major types of analysers:

1. **Continuous Flow**: In this type samples and reagents pass through a single or multiple sets of channels. The amount of sample and reagents is determined by the length and internal diameter of the tubing through which it flows. These types of analysers have now been replaced with other better systems.
2. **Discrete Analysers**: These are also called **Random Access** (RA) analysers. These consist of a system of moving cuvettes to receive samples and reagents from automatic dispensers or syringes. Various steps involved in the test procedure are almost the same as manual method. The samples may not be tested in sequence but can be programmed to have a user-defined sequence in order to have urgent or stat testing before the routine samples.
3. **Centrifugal Analysers**: In this system the contents of a single cuvette having partitions are mixed by centrifugal force generated by rotation of a rotor at high speed. Same cuvette or cell acts as reaction cuvette as well as measuring cuvette.

A detailed description of three common instruments is given below:

MICROLAB 200

Microlab 200 analyser is a semi-automated, manual, filter photocolorimeter. Microlab can be programmed for up to 60 different test methods. Once the parameters of a test have been defined, the information is displayed on the screen.



Optical path

The measurement takes place in a micro flow cell (reaction cup), which has a capacity of 32 µl. The reaction mixture is aspirated into the flow cell by a bellows pump. The sample volume is required in the range of 350–500 µl. The wavelength of light used for the measurement is selected by means of high quality narrow band interference filters, mounted on filter wheel. As a standard, six filters are mounted having wavelengths: 340, 405, 505, 546, 578, and 620 nm. The long-life quartz Iodine lamp is the light source.

External components

Sipper tube: It is a Teflon tube, located on the front of the instrument for the aspiration of the sample, operated by a metallic sipper button.

Sipper LED: The red LED (light emitting diode) along the sipper tube indicates the status of the pump action i.e., continuously on means aspirating, and flashing means dispensing to the waste bottle.

Keyboard: The keyboard consists of various functions key.

1. The four **hardware keys** are:
   a. ABS key: starts measuring the absorbance of solution present in the flow cell.
   b. Flush key: switches on the sipper pump in a continuous mode for flushing.
   c. Paper key: advances the printer paper to the desired position.
   d. Prime key: activates the sipper system for one cycle at the programmed volume.
2. Six **software function keys** are located just below the LCD (liquid crystal display) screen. These keys are used to select the information at the bottom of the screen display:
   a. Page key: change the display pages.
   b. Utility key: will return the system to utility menu.
   c. Skip key: move the programme

sequences in a forward direction and so on.

d. Alphanumerical keys: are used to programme the instrument and to select different commands on the display screen.
e. Clear key: to clear an entry during data entry.
f. Enter key: to confirm an entry via keyboard.

3. Besides these there are **cursor keys** of Back (<) & forward (>) space key to move back and forth during data entry.

Screen Display: The instrument has a high contrast liquid crystal display (LCD). It can show up to 16 lines of 20 characters each and can also show graphs.

Line/Power Switch: It is located on the back of the instrument and contains fuse to prevent high voltage surge of electricity.

Internal components

Light source: It consists of a 20 W/12V quartz iodine lamp.

Lens: It is placed between the light source and the filter for amplification and focusing light.

Filter: The colour (wavelength) of the light used for the measurement is selected by means of high quality, narrow band, interference filters. The instrument programme controls the movement of filter wheel and measurement can be either monochromatic or dichromatic and kinetic or end-point.

Cuvette: The measurement of absorbance takes place in the quartz micro flow cell. Its temperature is electronically controlled within a narrow range at 25, 30, or 37°C by means of peltier elements.

Photocell: The photocell measures intensity of light falling onto it and converts it into corresponding strength of electronic signals. The light sensitive layer deteriorates with the passage of time.

Motherboard: It is a printed circuit board on which integrated circuits (ICs) are mounted. The ICs are used for programming of parameters, calculation of results and for control of motor system.

Power supply: It is located under the motherboard and is shielded by a metallic cover. The power supply steps down line voltage.

Exhaust fan: It kept the temperature of power supply and the motherboard at an optimum level and prevents it from rising to a dangerous level.

SERVICE AND MAINTENANCE

Daily:

1. The Microlab 200 may be cleaned with a damp cloth. Make sure to remove the main plug before cleaning and take care that no water gets in to the instrument.
2. Check printer paper.
3. Empty waste container.
4. Flush the instrument thoroughly at the end of the test procedure and also at the end of the day.

As required:

Sometimes due to insufficient washing or long period of inactivity, the silicon tubing in the pinch valve gets blocked causing non aspiration of sample or leakage of liquid from the pressure relief system of the pump through the outlet at the bottom of the instrument. Proceed as follows:

1. Disconnect the instrument from power outlet.
2. Remove the top cover.
3. Stretch the silicon tube passing through the pinch valve and roll it between the fingers, trying to unstitch the tubing in the pinch area.
4. Check for the correct operation before mounting the top cover.

Internal tubing has to be replaced only once a year. However, tubing might get polluted due to lack of maintenance and may cause damage to the instrument.

SELECTRA-2

It is a random access, fully automated filter containing chemistry analyser based on principle of photometry. It can perform up to 150 tests per hour. Turn around time for enzymatic method is 5 min and for end point method is 11.5 min.

Components

Analyser Unit: It is composed of inbuilt computer, display, keyboard and main unit, which has sample tray, reagent tray and reaction rotor.

Display: It shows patient's data, analytes to be measured, results and quality control data. It has a memory and can store patient's results for up to 6 days and quality control data for up to 30 days.

Sample tray: It has a capacity of 60 samples, out of which 51 spaces are for routine samples, 3 are reserved for emergency (stat) and 6 for paediatric samples.

Reagent tray: It has a capacity of 30 reagents.

Sample Probe: It aspirates the sample from sample cup and places it in reaction rotor cell. It also mixes the sample with reagent in the cell. It is connected to Hamilton syringe through which

exact amount of sample to be aspirated is regulated, between 10-100 µl.

Reagent Probe: It aspirates the reagent and dispenses it in reaction rotor cell. A Hamilton syringe regulates the volume of the reagent.

Reaction Rotor: It is a rounded plastic plate in which 48 rectangular cells are present. Samples and reagents are mixed, incubated and measured in these cells or cuvettes. Ideally, it is to be replaced after 10,000 determinations, or when the baseline starts getting beyond the permissible range.

Cooling Unit: This unit is outside the main instrument. It provides coolant at a prefixed temperature, which circulates through the main unit to maintain it at a particular temperature.

Vacuum pump: This vacuum unit is placed outside the main analyser and it provides necessary suction for washing of cuvettes and dispensing the samples and reagents.

Printer: It is built-in in the instrument for printing results and quality control data.

Figure 6.3: Selectra 2 automated chemistry analyser

System operation

The analyser software has a menu-oriented structure. All functions can be called up from the main menu. Selection of menus and functions is carried out via display and keyboard. Each key has functions like request and load of samples, evaluation of samples and quality control. All these functions are given in the instruction manual.

Maintenance

The analyser does not have to be checked manually each day, i.e., all maintenance measures have only to be carried out periodically or whenever necessary. However, regular maintenance is specifically required to avoid unnecessary interruptions of the routine.

Daily

1. Empty and liquid waste container and refill the system wash solution.
2. Check the printed results of the cuvette blank measurement.
3. Prior to start of day's work check that there is sufficient amount of paper available in the printer.
4. Check that sufficient amount of HCl (cleaning solution) is present in the corresponding tube on the reagent rotor.

Weekly

1. Automated cleaning of the sample/reagent needle be performed.
2. Check syringes and Teflon tips for air bubbles.

Monthly

Clean the container for system liquid and waste with 0.1 N NaOH solution.

Trouble shooting

The analyser is an error-tolerant and user-friendly system. It not only informs the user on operating and system errors but also indicates their remedies by displaying messages. It warns the user about any irregularity found. Detailed information is available in instruction manual.

LIA-MAT-300

The LIA-MAT 300 analyser system is a fully automated, batch analyser for the *in-vitro* testing of hormones and tumour markers (PSA, α-fetoprotein, CA 125, CEA, etc).

Principle

It is based on two-site immunoluminometric assay (sandwich technique). The monoclonal antibody is used for the coating of the solid phase (coated tube), a second monoclonal antibody is used for the tracer. The tracer-antibody and the immobilised antibody reacts simultaneously with the analyte antigen e.g., CA 125, in the patient sample and the standard. Unbound material is removed by a washing step. The anti-CA 125-tracer conjugate consists of the monoclonal antibody and a covalently bound isoluminol derivative. The tracer-CA 125 complex bound to the tube wall in the immunological reaction is detected by a light reaction. Oxidation of the isoluminol is started by the automatic injection of alkaline peroxide and catalyst solution into the test tube. An immediate emission of photons occurs for few seconds in the luminometer. The light (425 nm) produced by the reaction is measured in the photomultiplier tube (PMT) of luminometer. The light signal measured in RLUs (relative light units) is directly proportional to the amount of CA 125 (analyte antigen) present in standard and sample.

Components

The instrument consists of an autosampler for

dispensing samples and reagents into test tubes placed in racks, a washing cum incubation unit, and a luminometer, besides standard components like Keyboard, Monitor and Printer.

Rack system: All the test tubes are placed in racks moving through various compartments of the instrument. Up to 28 racks can be loaded, each capable of holding 10 test tubes. The top of racks is marked with a notch. If the notch is on the right hand side, the No.1 test tube is on the right and No.10 test tube is on the left.

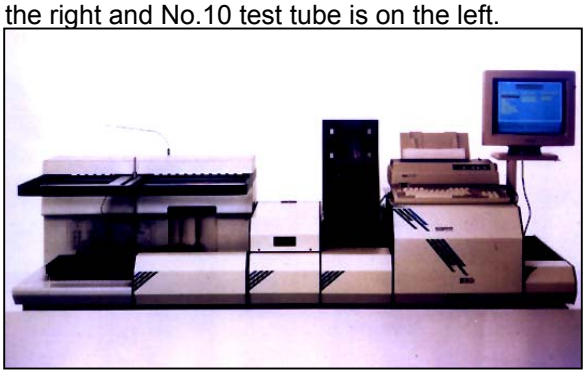


Figure 6.4: LIA mat 300 Autoanalyser system

Incubator: It allows the required assay incubation (binding of the tracer-antibody with analyte antigen in serum) at room temperature. After the racks are located, (housed) and automatically fixed, they are vibrated horizontally for the programmed time.

Washer: The washer runs the wash cycles consisting of aspiration and rinsing out of the leftover tracer and serum from tubes in racks. The racks must be fully loaded, if needed by empty tubes.

Measuring unit OR Luminometer with two Injectors: It measures the photon emission (light signal) in the form of relative light units (RLU). This signal is directly proportional to the concentration of the analyte.

Maintenance

1. **Monitors and Printer**: No specific maintenance is required for both these components except the standard care of any such part. Damp cloth with a mild detergent can be used to remove any dirt.
2. **Washer**: Check the wash quantity (liquid level) in the washer periodically to ensure that a constant level is maintained. Check the washer inlet filter each month for dirt and replace, if required. During replacement, ensure that the flow direction of the filter is correct. Remove any dirt using a damp cloth with a mild detergent.
3. **Incubator**: No specific maintenance is required for this unit. Damp cloth with a mild detergent can be used to remove any dirt.
4. **Cleaning the system**: Formation of crystals in the wash solution (NaCl) with subsequent sticking of the tubing system can be avoided by repeated rinsing at the end of each session. Additionally, following maintenance routines need to be performed once every week or more frequently if needed:
   a. Carefully wipe off pipetting needle with alcohol.
   b. Check rinsing pumps for water tightness.
   c. Wipe off supply lines with moist cloth.
   d. If necessary, remove grease with alcohol.
   e. Clean surfaces of manifolds

Special precautions

1. Reagents contain sodium azide as preservative so; avoid contact with skin and mucous membrane.
2. After discarding the waste, it should be flushed with copious amount of water so that azide does not react with lead waste pipes.
3. Mouth pipetting, smoking, eating and drinking water are not allowed while handling the reagents. One needs to wear gloves throughout the testing procedure.
4. Hand washing after completion of the test procedure is mandatory.

AUTOMATION IN HISTOPATHOLOGY

Tissue processing and staining techniques in histopathology involve a number of steps. At each step the reagent and timing vary and in some even the temperature is different. In a laboratory dealing with a large number of specimens it is difficult to deal with all of them by manual techniques. This department has also benefited from the recent advances in technology. Introduction of automated equipment in tissue processing and staining has greatly facilitated the handling of heavy workload. The equipment is described in the section on Histopathology (page 385).

7. QUALITY CONTROL

QUALITY CONTROL

The term quality control refers to the steps taken by the laboratory to ensure that the tests are performed correctly. It is primarily aimed at minimising the analytical errors but this alone may not be sufficient because there are factors operating outside the laboratory, which may affect the report produced by the laboratory. These include non-analytical errors, advice by the clinician, sample collection, transport, collection and storage of specimens at the laboratory reception and preparation and despatch of laboratory reports. This will require additional measures to control non-analytical errors.

QUALITY ASSURANCE

This describes all the steps taken both in and out side the laboratory to ensure that the results are correct and reliable. The factors affecting quality assurance include:

1. The selection and quality of specimen containers, preservatives and anticoagulants.
2. Method of specimen collection.
3. Specimen labelling.
4. Completion of laboratory request forms.
5. Transportation of specimen to the laboratory.
6. Recording and labelling in the laboratory.
7. Skill of the technicians performing the tests.
8. Quality of reagents, equipment and laboratory ware.
9. Quality of method employed.
10. Controls employed to check the analytical process.
11. Method of reporting of the results.

TERMINOLOGY IN QUALITY CONTROL

Accuracy
The accuracy of an analytical measurement is how close a result comes to the true value (Figure 7.1). It is the degree of agreement between observed and true value of a constituent in the specimen. Determining the accuracy of a measurement usually requires calibration of the analytical method with a known standard (page 47).

Precision
It is defined as the degree of agreement between replicate measurements of a constituent in a specimen (Figure 7.1). It is the measure of reproducibility of test results.

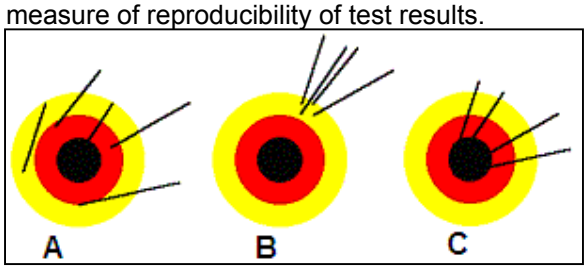


Figure 7.1: Illustration of accuracy and precision. (A) No Accuracy, No Precision; (B) Precise but No accuracy; (C) Accurate and precise

Specificity
It is the ability of an analytical method to determine exclusively the analyte it claims to measure without reacting with other related substances.

Sensitivity
It is the ability of an analytical method to produce a change in signal relative to a change in quantity, concentration or property of the analyte.

Interference
The term interference describes the effect that a compound or group of compounds other than the analyte in question has on the accuracy of measurement of an analyte.

Detection limit
It is the ability of the method to detect the lowest concentration of a constituent in a specimen.

LABORATORY ERRORS

Two types of errors are encountered in clinical laboratories:

1. **Errors of scatter (imprecision) or Random errors**: These are irregular or random errors and irreproducibility in making replicate measurements. These affect the precision of a result. The results differ from the correct values by varying amounts. These errors result from following factors:
   a. Faulty technique including incorrect and variable pipetting, inadequate mixing of sample with reagents or incubation of

tests at inconsistent temperatures for incorrect length of time.
   b. Dirty glassware
   c. Heavy workload resulting in faulty technique or shortcuts being taken.
   d. Too low workload resulting in loss of concentration and errors being made.
   e. Fluctuating voltage.
   f. Interfering substances e.g., haemolysis, lipaemia and icterus.
2. **Errors of bias (inaccuracy) or Systematic errors**: These are consistent, regular or fixed errors. All results differ from the correct result by approximately the same amount. These errors result from biases introduced by instrumental method, or human factors The common causes of inaccuracy are due to following factors:
   a. Use of unsatisfactory reagents that contain impure chemicals prepared wrongly, stored incorrectly or used after their stated expiry date.
   b. Incorrect or infrequent calibration of test method or the instrument.
   c. Use of control sera that have been wrongly prepared, incorrectly stored or have expired.
   d. Test being read at incorrect wavelength.

STANDARDS

Analytical Standards

Standards are materials containing a known concentration of an analyte. They provide a reference to determine unknown concentration or to calibrate an analytical instrument. Determining the accuracy of a measurement usually requires calibration of the analytical method with a known standard. This is often done with standards of several concentrations to make a calibration or working curve (see PREPARATION OF CALIBRATION CURVE on page 47). A standard has following criteria:

1. It must be a stable substance of known composition.
2. It must be capable of drying at 105-110°C without any change in the composition.
3. It should have high equivalent weight so that weighing errors have small effect.
4. It must be capable of accurate analysis.
5. Desired reaction should occur rapidly and completely.
6. Its purity must have been assured.

Standards can be of the following types:

- **International Standards**: When recognised by an international body as WHO and are assigned a unit by it.
- **National or regional Standards**: These are prepared at national or regional level and are comparable to international standards.
- **Local Standards**: These are prepared locally and calibrated against the international or national standard.

Depending upon their quality the standards may be of following types:

Primary Standards

A primary standard is a reagent that is extremely pure, stable, has no waters of hydration, and has a high molecular weight. See Appendix V: Preparation of standard solution of acids and bases on page 418 to prepare approximate 1N solutions of some common acids and bases.

Some primary standards for titration of acids are:

   a. Sodium carbonate: NA_ACO, mol wt 105.99
   b. Tris-(hydroxymethyl)aminomethane (TRIS or THAM): $(CH_2OH)_3CNH_2$, mol wt. 121.14).

Some primary standards for titration of bases are:

   a. Potassium hydrogen phthalate (KHP): $KHC_8H_4O_4$, mol wt. 204.23
   b. Potassium hydrogen iodate: $KH(IO_3)_2$, mol wt. 389.92

Secondary Standards

A secondary standard is prepared in the laboratory for a specific analysis by standardisation against a primary standard.

CONTROLS

Control material is used for quality control purposes. The concentration of analyte is not as accurate as is required for a standard as a target value and a range is usually mentioned. Therefore, It cannot be used for calibration. The matrix of control material needs to resemble the biological specimen with which these are analysed. Control material is to be inserted in a batch of tests at frequent intervals and the results are evaluated statistically to ensure the quality of test procedure, equipment used and result produced. Controls are used to study precision and changes in accuracy.

STATISTICS IN QUALITY CONTROL

Mean ($\bar{X}$)

It is defined as, the average of a series of values determined by a given method. It is calculated by the formula:

$$\overline{X} = \frac{\sum x_1 \ldots x_N}{N}$$

Where $\overline{X}$ = mean (pronounced as x bar)
x = individual values from x_1 to x_N
N= Number of observations

Standard deviation (SD)
It is a measure of deviation or scatter from the mean in a series of values. It is a statistical measure of the precision in a series of repetitive measurements and denotes confidence limits. Standard Deviation is calculated by the formula:

$$SD = \sqrt{\frac{\sum_{i=1}^{N}(x_i - \bar{x})^2}{N-1}}$$

Where *SD* is standard deviation, *N* is the number of observations, x_i is each individual measurement, and $\bar{x}$ is the mean of all measurements. All modern calculators provide this function. Otherwise it can be calculated by:

- Sum and square the differences of all the values from mean.
- Divide it by n-1
- Take the under root.

Variance
It is the square value of the standard deviation from the mean and is calculated by:
Variance = SD^2

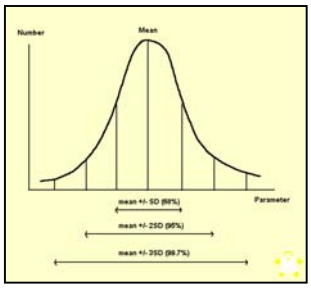
Coefficient of Variation (CV)
It is a measure of variability around the mean expressed in percentage. It is also a measure of scatter around mean but in percentage. Thus:

$$CV\,(\%) = \frac{SD}{Mean} \times 100$$

Standard error
It is another expression of variance or scatter calculated by: $\frac{SD}{N}$

Confidence Limits
This is defined as percentage certainty with which values in a series will lie within a given range. This is usually expressed as mean ±2SD. A single SD value gives 68% confidence limit while ±2SD gives about 95% confidence limit.

Distribution
Raw data is given on an interval scale showing the occurrence of data at each interval. This is **frequency of distribution**. If the same is made on the graph paper and upper ends of each line are joined a distribution curve is formed. If the curve is bell shaped it is called normal or **Gaussian** distribution. In this curve the series is symmetrically distributed on either side of the mean value.



TYPES OF QUALITY CONTROL PROCEDURES

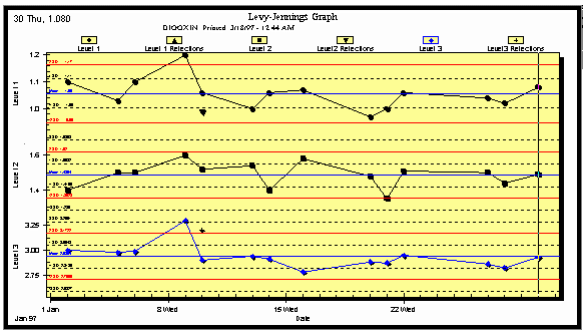
There are two types of quality control procedures:

- Internal Quality Control, and
- External Quality Assurance

INTERNAL QUALITY CONTROL

This includes all quality control procedures adopted by the laboratory staff for checking day-to-day accuracy and precision of all procedures and equipment.

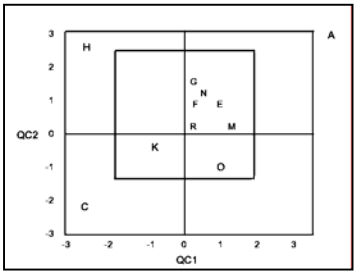
1. **Levy-Jennings Chart**: Consecutive daily measurements of controls are used to calculate the mean and SD value. These values are plotted on a graph paper showing horizontal lines of mean ±1SD, ±2SD and ±3SD. The Levy-Jennings chart is used to



evaluate accuracy and precision. If precision remains unchanged then 68% fall within ±1SD and 95% values fall within ±2SD range. They are scattered evenly on both sides of the mean. Permissible limits are one in 20 readings crossing 2SD limit and 3 in 1000 readings crossing 3SD limit. The values of controls (normal and abnormal) are plotted daily on the LJ chart and these values are monitored using Westguard rules, explained below:
   - o 1,2 SD: One control observation exceeds control limit set at ±2SD is a warning sign.
   - o 1,3 SD: One control observation exceeds ±3SD is a random error subject to rejection rule.
   - o 2,2 SD: Two consecutive control observations exceed ±2SD is a systematic error subject to rejection rule.
   - o R4 SD: One control observation exceeds the +2SD and the second

control observation exceeds -2SD is a random error subject to rejection rule.
- o 4,1 SD: Four consecutive readings crossing ±1SD on one side is a systematic error subject to rejection rule.
- o 10x: Ten consecutive control readings on one side of the mean is a systematic error subject to rejection rule.

2. **Five Cycle Quality Control Charts**: It is designed to facilitate the plotting of daily results. Control runs are repeated 5 times to provide a wide range scale of values. Small numbers to the right assist in identifying correct points for plotting the result. Horizontal lines are drawn at the mean and ±2SD values of reference material or control. Control is inserted randomly in daily runs and mean is calculated at the end of the day. This is plotted on the graph. There is only 1:32 chance that 5 results in a row will be either above or below the mean value. If it occurs, this is called upward or downward shift. It may be due to change in reagent concentration, change in equipment calibration or deterioration of reference material. If test value keeps on decreasing or increasing on one side of the mean it is called upward or downward trend. It results from gradual change in calibration or concentration or apparatus, reagents, reference material or equipment.
3. **Moving Two Standard Deviation Charts**: In these charts there are 4 columns. Left side column is for entering daily change from mean assigned to a control. Mean of control run during the day is calculated and its difference from assigned mean is deduced. It is entered in front of day number disregarding plus or minus sign. At the end of week the values are summed and divided by two. This is 2SD. Assigned mean is written on top and 2SD value is entered in the second column. In the third column it is entered and divided by number of week (1 for first week). Deduced value is then entered in the third column. This will be 2SD for next week. In second week 4th column value of previous week is summed with that of current week in 4th column. In third week 4th column value of previous week is summed with second column value of current week in 3rd column divided by three. This value is entered in 4th column. 2SD value in each week should not differ significantly from assigned 2SD value of reference material or control. If it happens check reagents, technicians performance, accuracy and calibration of apparatus and equipment.
4. **Youden Plots**: These are designed to compare results on two controls. Result for one is plotted on one axis and for second on the other. Zero lines



are drawn for both and so are the lines for assigned ±2SD values. A line is drawn joining all crossing points of lines with zero angles. Squares defined by 2SD on either side of zero line should enclose 95% of all the results.
5. **Use of results on patient's sample**: Selected patient's sample from previous day can be run to check between run precisions. Daily patient's results lying within a defined limit are selected and mean is calculated. It should be relatively constant. This will be affected by laboratory errors. It is also sensitive to non-analytical errors such as errors in sampling and transportation. These can be plotted serially on a graph.
6. **Cumulative Sum (Cusum) Charts**: This can be applied to daily means or to values of controls. It is plotted in such a way that SD value of each day is added to or subtracted from the previous day's result to obtain cusum plot rather than plotting with regards to mean every day. Cusum values plotted will tend to remain in a straight line as long as accuracy is not changed.

EXTERNAL QUALITY CONTROL

This is to integrate performance of different laboratories so that results are mutually interpretable. Samples to be analysed by standard methods are distributed to all participating laboratories for analysis. Results are then subjected to statistical analysis. This also enables comparison of different methodologies used by different laboratories and recommendations for standard methods can be made.

8. SPECIMEN COLLECTION AND TRANSPORT

The collection of specimens for laboratory tests from patients consists of following steps:

- Documentation/Registration of the patient
- Collection of specimen
- Dispatch of specimen to respective department

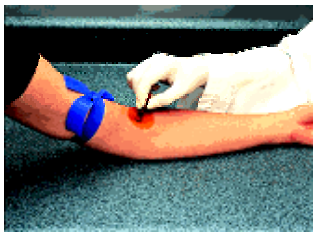
DOCUMENTATION AND REGISTRATION

Patient reports to the reception desk. The reception staff registers the patient and documents his/her identification and demographic data consisting of Regt/Hospital No, Rank/designation, Name, Age, Unit/Address and the tests to be carried out for that particular patient. Reception staff checks the entitlement of the patient by means of family treatment card/ unit certificate/ individual's identity card /discharge/release documents etc. Patient is provided with a receipt having details of the tests to be carried out and the tentative delivery date for the complete lab report. He is requested to sit in the waiting area to wait for his/her turn for specimen collection.

COLLECTION OF SPECIMEN

BLOOD SPECIMEN

1. Make the patient to sit comfortably in the phlebotomy chair. Identify the patient by asking his particulars and compare them with the request form.
2. Inform the patient about the specimens to be collected. Always ask if he or she has undergone blood tests previously. In case of any history of abnormal reactions to blood collection, inform MO I/C lab/Pathologist before phlebotomy and then follow his instructions.
3. Thoroughly check the request form for the number and type of the investigations. Prepare proper labels and paste them on appropriate containers before obtaining specimens. In case of any doubt, check the authenticated test list where information regarding type, quantity, preservative and storage of the specimen is given for various blood tests. If still there is any doubt, ask the senior colleague/NCO/JCO in-charge or the Pathologist.
4. Select syringe of appropriate size so that the quantity of blood required can be obtained in single prick. If multiple samples are required, or >15 ml of blood is to be collected use a butterfly needle or a canula.
5. Select appropriate vein (preferably antecubital) from forearm. Cleanse the skin over the venepuncture site in a circle approximately 5 cm in diameter with 70% alcohol/spirit swab, scrubbing the area vigorously.
6. If the sample is to be collected for blood culture then skin is to be thoroughly sterilised rather than simple cleansing. Follow the procedure as under:
   a. Starting in the centre of a circle apply 2% iodine (or povidone-iodine) in ever-widening circles until the entire chosen area has been saturated with iodine.
   b. Allow the iodine to dry on the skin for at least 1 min.
   c. Completely remove[1] the iodine with 70% alcohol/spirit swab following the pattern of application.
7. Apply a tourniquet tight enough to obstruct venous flow only and relocate the vein to be punctured but don't touch the proposed site of needle entry or the needle itself. Ask the patient to clench the fist to make the veins prominent. If the vein is not visible, palpate it with fingers. In case the veins of forearm are not visible/palpable, other sites such as dorsum of the hand may be selected.



8. Insert the needle into the vein and withdraw blood till the required quantity of blood is obtained. Do not try to withdraw the piston too forcefully (hard pulling) as it can collapse the vein and it may cause frothing/ haemolysis of the specimen.
9. Release the tourniquet once the needle has entered the vein.
10. Apply pressure with thumb on antiseptic swab at puncture site for 2-4 min till the blood ooze stops. Only then patient should

[1] This is important to wipe of iodine to prevent iodine sensitisation.

be allowed to move away from the specimen collection chair. The antiseptic swabs should be disposed off in designated baskets.
11. Remove the needle from the syringe.
12. The blood from syringe is distributed to appropriate, labelled containers.
13. Inform the pathologist promptly under the following circumstances:
    a. If patient feels unwell after specimen collection, ask him to lie down on couch, reassure and give him hot drink.
    b. Some patients collapse when the skin is punctured or at the sight of blood. In such cases withdraw the needle immediately and ask the patient to lie down in supine position. Raise the legs of the patient.
    c. If specimen is not drawn in first prick.
    d. In case of children below the age of one year.
    e. In case of very sick patients/special blood specimen collection.

Blood specimen for serology

Serological tests are required in most of the bacterial, viral and parasitic diseases. A clotted blood specimen is preferred.

1. A vacuum collection system is both convenient as well as reliable.
2. Paired specimens are to be collected during acute and convalescent phases of illness in certain viral and other infections to document a diagnostic rise in antibody titre (see VIRAL SEROLOGY on page 204).
3. Protect blood specimens from extremes of heat and cold during transport.
4. Specimens must be refrigerated. Whole blood is to be stored at 4°C. Serum can be frozen at -20°C or lower temperature and can be sent frozen to the reference laboratory.
5. Sera for serology cannot be kept below 0°C, instead should be kept at 2-8°C.

Blood specimen for culture

1. Contact microbiologist/pathologist for appropriate media for blood culture, as the media may vary depending upon the type of pathogen suspected.
2. Wash the hands with soap and water and wear sterile gloves.
3. Withdraw the blood following the procedure described above.
4. Change needle before injecting the blood into the culture bottle.
5. Thoroughly clean the rubber bung of the culture bottle with iodine solution and inject an amount of blood equal to 10% of the volume of medium (for 30 ml medium 3 ml blood and for 50 ml medium, 5 ml blood is needed).
6. After the needle has been removed, the site should be cleaned with 70% alcohol/spirit swab again.
7. Don't store the containers and caps separately.
8. Blood obtained for culture of suspected anaerobes should not be exposed to air in any way.

CULTURE SPECIMEN - GENERAL CONSIDERATIONS

1. As far as possible specimens for culture should be obtained before administration of antimicrobial agents.
2. If it is not possible then the laboratory should be informed about the therapeutic agent(s) so that this fact is considered before issuing laboratory report.
3. Material should be collected from the appropriate site where the likelihood and possibility of isolation of suspected organisms is high.
4. Sometimes patient's active participation is necessary for sample collection (sputum or urine), so he should be instructed properly and accordingly.
5. Sufficient quantity of specimens is to be collected to permit complete examination.
6. Specimens are to be placed in sterile containers.
7. Some specimens are directly collected in culture media. Contact laboratory if such collection is required.
8. Proper labelling of specimens should always be done with patient's name, test type, date and site of collection etc.
9. The relevant clinical information is to be recorded on the request form.
10. Any condition, circumstances or situation that will require special procedures should also be noted on the request from.
11. Specimens should be collected during working hours except in emergency, so that the services of qualified microbiologist will be available to directly supervise processing of the specimen.
12. The most appropriate specimens for isolation of viral, chlamydial or rickettsial agents depend on the nature of the illness.
13. The material should be collected as early as possible in the acute phase of the disease, because these agents tend to disappear relatively rapidly after the onset of the

symptoms.
14. Vesicle fluid is preferably collected in a syringe or capillary pipette and immediately diluted in an equal volume of skimmed milk or tissue culture medium.
15. All specimens for viral culture should be frozen and stored at -70°C until culture is initiated.

THROAT AND NASAL SWAB

1. Throat swab cultures are to be taken under direct vision with good light.
2. Areas of exudation, membrane formation, any inflammation or if not seen then tonsillar crypts are the sites of choice.
3. Nasopharyngeal swabs are better taken by treating physician/surgeon himself.
4. For recovery of viral agents, washings are collected after gargles with nutrient broth by the patient.

NASAL SPECIMEN FOR *Mycobacterium leprae*

The nasal specimen for *M.leprae* can be taken as follows:

Nasal swab
1. Make the patient sit with his head bent backwards but facing the light.
2. Insert and repeatedly rotate the swab into one of the nasal cavities, against upper part of the nasal septum.
3. Make 2-3 evenly spread smears.
4. Air dry the slides, wrap in a paper and send to the laboratory.

Nasal washings and nasal blow
1. Make the patient sit. Place a few drops of sterile saline in the nose.
2. After 3 min, ask the patient to blow hard his nose on a small sheet of plastic or cellophane. (This plastic or cellophane can be given to the patient to take it home and ask him to blow hard onto the sheet, the following morning, soon after waking and before washing. The patient can bring it directly to the laboratory).
3. Transfer some of the mucus pieces from the washing to a slide with a clean wooden stick and make thin smear.
4. Air dry slide and send it to the testing area

SPUTUM SPECIMEN

1. Early morning specimen is preferred.
2. Give the patient a clean, dry, wide necked, leak proof container.
3. Patient should cough deeply to produce sputum.
4. For *M.tuberculosis* culture, a series of three fresh, early morning specimens (5-10 ml) are collected and kept in the refrigerator. If amount is less, the patient is advised to collect 24 h sputum or until 50 ml is obtained.
5. *M.tuberculosis* can be recovered from the gastric contents in infants, debilitated patients and those who are unable to co-operate in the collection of sputum. This can be obtained by gastric aspiration performed as an indoor procedure.
6. Gastric washings are better collected early in the morning, in fasting state. These are neutralised soon after collection by N/10 NaOH.

FAECAL SPECIMEN

1. Rectal swabs are often helpful in identifying the cause of acute bacterial diarrhoea when stool specimen cannot be collected readily.
2. Faeces should be passed directly into a clean, waxed cardboard container that is fitted with a tight cover.
3. Residual soap/detergent, disinfectant in the bedpan or faeces contaminated with urine may make it unsatisfactory.
4. Faeces obtained are transferred to another clean container. The specimen should include any pus, blood, mucus or formed elements that may have passed with stool and should include the representative fraction of the first, last and middle portion of the faeces
5. Specimen (~1 ml) is added to 10 ml sterile alkaline peptone water in suspected cholera cases.
6. If viral infection is suspected the faeces are extracted with sterile buffered saline. Faeces (~1 ml) are mixed with 9 ml sterile buffered saline, allowed to sediment for 30 min (or centrifuged). The supernatant is transferred to a sterile container, frozen and kept below -40°C until processed. (Paired sera are also to be collected at the same time and after 2-3 weeks).

URINE SPECIMEN

Urine specimen is often collected by patient him/herself. Therefore, the patient needs to be properly instructed to have correct sample collection. An uncontaminated mid-stream urine (MSU) sample is the best and following methods are to be used for its collection:

Females
1. Wash the genital area thoroughly with soap and water (may be omitted for urine RE).

2. With two fingers of one hand, hold the outer folds of vagina (labia) apart. With the other hand, rinse the area from the front to the back with soap and running tap water.
3. Start urination so that the stream of urine should flow without touching the skin. After a few moments, place a sterile container under the stream of urine. Remove it from the urine stream the moment required amount of urine is collected.
4. Secure and tighten the cap on the container.

Males

1. Wash the genital, area thoroughly with soap and water (may be omitted for urine RE).
2. Start urination and after a few moments, place a sterile container under the stream of urine. Collect the required amount of urine and remove the container from urine stream.
3. Secure and tighten the cap.

Infants, uncooperative and debilitated patients

1. Plastic bags may be attached after careful and thorough washing of the genital area.
2. The bags should be watched so that they can be removed immediately after patient has passed the urine.
3. If the patient has not voided urine within 30 min the collecting bag is removed.
4. Patient needs to be re-scrubbed and a new collection device is to be attached.

Urine collection for *Mycobacterium tuberculosis*

1. Three consecutive early morning specimens (>90 ml each) collected in sterile container are superior to 24h collection.
2. Boric acid (1.6%) is used as preservative in case of 24h urine collection in exceptional situations e.g., when patient cannot report daily for sampling.
3. Suprapubic aspiration in ward by a doctor is preferred in catheterised patients.

SPECIMENS FOR HISTOPATHOLOGY

GENERAL CONSIDERATIONS

1. Container must be several times larger than the specimen.
2. It should be wide mouthed and flat-bottomed.
3. It should have a screw cap.
4. The plastic container is always preferred over the tin jar.
5. It should have perpendicular walls.
6. Always avoid using the empty tin of casting plaster or any other material as a container.
7. Container should have a label with name of the patient, bed number, ward and nature of specimen.
8. The surgical specimen should be washed with tap water to remove extra blood whenever possible.
9. The large specimens may be incompletely sliced with sharp knife for better fixation.
10. The accompanied request form should have name, age, ward, site of biopsy and brief clinical history. X-rays should accompany bone specimens.

FIXATIVES

1. In routine, 10% formal saline is an appropriate fixative. It is prepared by diluting one part of 40% formalin in nine parts of physiological saline. Pure formalin (40%) should not be used because it hardens the specimen.
2. Specimens for frozen section are sent in physiological/isotonic saline.
3. Bone marrow trephine biopsy is fixed in Zenker's solution/formalin or any suitable fixative.
4. Post-mortem specimens are fixed and transported in 10% formal saline.
5. The quantity of fixative should be 3-4 times the size of the surgical specimen.
6. In special situations always consult pathologist about the fixative to be used. (See also the section on COLLECTION OF BIOPSY SPECIMENS on page 379).

SPECIAL SITUATIONS

Whole lung: Wash with normal saline. Inject fixative in the major bronchus. Immerse it in a wide mouthed jar containing enough fixative.

Large Cysts: Puncture the cyst wall. This will drain its contents and will reduce the size. Place it in a container of appropriate size with fixative. The request form must contain information regarding the amount and nature of fluid drained.

Limb: Amputated limb is washed. Fixative is injected in a major vessel, until no more fixative can be injected. Wrap the whole limb in a muslin cloth soaked and place it in a container filled with fixative.

Lymph Nodes, Glands etc. These specimens are carefully split in the middle and placed in fixative.

Skin/muscle biopsy specimen: The excised piece of skin is placed flat on filter paper to drain out the extra blood and put in a fixative (10% neutral buffered formalin).

Post-mortem Specimen

Each representative section is separately placed in a gauze piece. Double label made of paper is stitched to gauze. All specimens are placed in a single, properly labelled container.

Whole brain: To keep brain's shape and gross anatomy intact the following procedure is recommended for fixation:

1. Wash the brain with normal saline.
2. Inject 10% formal saline into basilar artery.
3. Fill half of the bucket with 10% formalin.
4. Pass a strong linen thread through basilar artery and tie both ends to the hooks of bucket. This will make the brain float in the bucket. The bucket should have enough fixative so that brain can float freely in it.

SPECIMENS FOR CYTOLOGY

General considerations

1. The specimen needs to reach the laboratory without any delay. If delay is expected, keep the specimen in refrigerator.
2. Add a fixative to the container before collection of specimen.
3. Commonly used fixatives are:
   a. Ethyl alcohol 95%
   b. Ether-Alcohol: Add equal mounts of ether and 95% alcohol.
   c. Add anticoagulant in the fluid specimen if high protein content is expected. An ACD bag is preferred.

(See also the section on COLLECTION OF CYTOLOGY SPECIMENS on page 380).

HANDLING OF INFECTIOUS SAMPLES

Laboratory staff is often confronted with the problem of handling highly infectious samples from patients such as viral hepatitis, HIV, rabies etc. Following must be observed for personal (self) protection:

1. Phlebotomist must wear gloves before venepuncture.
2. He should exercise due care to prevent spillage/splashes while transferring blood to containers from syringe.
3. The blood container be labelled with red marker as ***Infected Material*** and make it air tight. Red stokers are to be pasted on the request forms.
4. Respective departments carrying out the test must be informed about the infective nature of specimens.

GENERAL CONSIDERATIONS FOR TRANSPORT OF SPECIMEN

1. All biological specimens must be considered hazardous and infected.
2. Exterior of the container should not be soiled/contaminated with the specimens.
3. Sufficient absorbent materials must be used to pack the specimen, so that it absorbs the spilled liquid in case of leakage/breakage during transit to reference laboratory.
4. Specimen containers must be leak proof and unbreakable. Plastic containers are preferred.
5. Specimens must be promptly delivered to the laboratory for valid results.
6. Some culture specimens require **transport media** (see TRANSPORT MEDIA below for details).
7. Specimens are to be refrigerated or incubated at 37°C, as the case may be, if there is a delay in transport of specimens to laboratory.
8. An appropriately filled request form should always accompany all specimens to guide the pathologist in selection of suitable media or appropriate technique.

DISPATCH OF SPECIMENS FROM RECEPTION TO INSIDE THE LABORATORY

1. Match the containers and respective request forms, number them and enter in the dispatch register/computer. Verify while handing over/taking away to respective department of the laboratory.
2. Notify the concerned department about urgent and special tests.
3. Inform the pathologist about any important specimen.

TRANSPORT MEDIA

Although transport media are useful, they remain second best to processing clinical material immediately after it is collected. A number of systems have been devised to reduce the effect of desiccation on swab and to dilute inhibitory substances in the swabs or in the clinical material itself. Nutrient broth is not satisfactory in that commensals may multiply in it and overgrow fragile or delicate pathogens. Although most such transport or holding media were originally designed to ensure survival of gonococci, other microorganisms also survive quite well. Some kind of holding or transport medium must be used whenever delay in transport to the laboratory is anticipated. Although these are commercially available, but can be prepared in-house as described below:

Cary-Blair transport medium

Sodium thioglycollate	0.75 g

Disodium hydrogen phosphate Na_2HPO_4)	0.55 g
Sodium chloride	2.5 g
Agar	2.5 g
Calcium chloride 10g/L (1% w/v)	4.5 ml
Water	495 ml

1. Dissolve dry ingredients by heating.
2. Allow to cool to 50°C and add 4.5 ml freshly prepared calcium chloride solution. Mix well.
3. Adjust the *p*H to 8.4 by 0.1 M (N/10) NaOH.
4. Dispense 7 ml in screw cap bottles of 9 ml capacity.
5. Sterilise by steaming for 15 min.
6. These bottles can be kept for 6 months.

Uses: Useful for preservation of enteric pathogens. It is also a good transport medium for *Yersinia pestis* (Plague bacillus).

Amies transport medium

Charcoal Pharmaceutical, neutral	10.0 g
Sodium chloride	3.0 g
Sodium hydrogen phosphate	1.15 g
Potassium dihydrogen Phosphate	0.2 g
Sodium thioglycollate	1.0 g
Calcium chloride	0.1 g
Magnesium chloride	0.1 g
Agar No.1	4.0 g
D/Water	1000 ml

1. Dispense well-mixed medium in screw capped Bijou bottles.
2. Sterilise by autoclaving at 121°C for 15 min.
3. Bottles can be kept for 9 months.

Uses: It is used for transport of specimen suspected to have anaerobes, urethral and other genital area specimens and sputum.

Stuart transport medium

Sodium glycerophosphate	10 g
Sodium thioglycollate	0.5 g
Cysteine hydrochloride	0.5 g
Calcium chloride	0.1 g
Methylene Blue	0.001 g
Agar No.1	5.0 g
D/Water	1000 ml

Mix ingredients and fill small Bijou bottles. Sterilise at 121°C for 15 min.

Uses: It is used for transport of urethral and other genital specimens, sputum and throat swab for *Corynebacterium diphtheriae* and *S.pyogenes*.

Glycerol saline transport medium

Sodium chloride	4.2 g
Disodium hydrogen phosphate (anhydrous)	3.1 g
Potassium dihydrogen phosphate	1.0 g
Phenol Red 1% (w/v)	0.3 ml
Glycerol	300 ml
D/Water	700 ml

1. Dissolve dry chemicals in water and adjust *p*H to 7.2.
2. Add phenol red solution and glycerol. Mix well and dispense in 7 ml amount in screw-capped bottles.
3. Sterilise at 121°C for 15 min.
4. Bottles can be stored at 2-8°C for 2 years.

Uses: It is used to preserve enteric pathogens like Salmonellae, Shigellae and *E.coli* etc. It is not suitable for *V.cholerae*, *Campylobacter* sp, or *Y.enterocolitica*.

Alkaline peptone water

Peptone	5 g
Sodium chloride	5 g
D/water	500 ml

Dissolve ingredients, adjust *p*H to 8.6-9.0 and dispense in 10 ml screw capped bottles. Sterilise at 121°C for 15 min. Bottles can be kept at 2-8°C for 2 years.

Uses: It is used for transport of faecal specimens for *V.cholerae* and other vibrios.

Virus transport medium

Hank's balanced salt solution	43.0 ml
Bovine albumin 100g/L (10% w/v)	5.0 ml
Phenol Red 4g/L (0.4% w/v)	0.25 ml
Nystatin (2500 IU/ml in sterile PBS[1])	0.5 ml
Penicillin (104 IU/ml and Streptomycin 10 mg/ml in sterile PBS)	0.5 ml

1. Add aseptically the sterile bovine albumin, phenol red, nystatin, penicillin and streptomycin solution to the sterile Hank's balanced salt solution. Mix well after each addition.
2. Adjust *p*H to 7.0
3. Dispense aseptically in 2 ml amount in sterile screw capped bottles.

Uses: Various viral specimens for culture can be sent in this medium (see also VIRUS ISOLATION on page 205).

Bordetella transport medium

Sterile sheep or horse blood	10 ml
Cephalexin (40 mg/L)	0.4 ml

1. Prepare and sterilise charcoal agar as instructed by the manufacturer (half strength). Transfer to a 50°C water bath.
2. Add aseptically the sterile blood and mix gently.
3. Add antibiotic solution and mix gently. Avoid formation of foam or froth.
4. Dispense in sterile 5 ml capacity Bijou bottles
5. It can be kept for 8 weeks at 2-8°C.

Sucrose buffer for transport of specimen with suspected chlamydiae infection

Stock solution

[1] Phosphate Buffered Saline

Ingredient	Amount	Water
Sucrose	68.5 g	100 ml
Dipotassium hydrogen phosphate (K_2HPO_4)	2.1 g	60 ml
Potassium dihydrogen phosphate (KH_2PO_4)	1.1 g	40 ml

Mix all the three solutions and make volume to one litre with distilled water. Boil for 30 min. Cool to room temperature.

Working solution

1. In 100 ml stock solution, add 10 ml foetal calf serum, 2 mg Gentamicin powder, 0.5 mg Amphotericin B powder and 10 mg Vancomycin.
2. Dispense in 1 ml amount into sterile screw capped plastic disposable test tubes.

SECTION II - CLINICAL PATHOLOGY

9. URINE EXAMINATION

Man has been curious about urine from the earliest times. The Babylonians and Sumerians studied urine and attempted to relate its appearance to various human ailments. Early Hindu literature describes “honey urine” as one, which attracted ants. Primitive analysis included tasting of urine. The early 19th century marks the start of scientific methods of urine examination. In 1827, Richard Bright studied urine at Guy’s hospital. In 1909, Stanley Benedict described solution for quantitation of glucose in urine. In 1941, Dr. Walter Compton created clinitest, for the measurement of reducing sugars. This test was the forerunner of the wide range of convenient urine tests to be used all over the world.

Urine testing provides rapid, valuable and reliable information into the health status of the patient. Urine is a valuable index to many normal and pathologic mechanisms. It is a complicated aqueous solution of various organic and inorganic substances. These substances are products from the body metabolism (either normal or abnormal) or products derived directly from foods.

Many urine characteristics and components are unstable. The urine is also an excellent culture medium. Therefore, all specimens should be examined within 30 min of collection or samples should be refrigerated. The delay in testing may result in gross changes, which affect the test results. Bacterial action affects pH, glucose, ketones and RBCs. Hydrolysis and oxidation affect bilirubin. Delay and exposure to light results in photo-degradation of urobilinogen to urobilin and volatilisation of acetone. It should be noted that sediments are unstable even at reference temperature if the urine is alkaline.

Major sources of error are:

1. Bacterial or chemical contamination.
2. Contamination with menstrual blood.
3. Contamination with vaginal and urethral discharges.
4. Inadequate mixing before examination.
5. Wrong/inadequate preservative

TYPES OF URINE SPECIMEN

First morning specimen
It provides concentrated urine as the bladder incubated it the whole night. It is best for nitrite, protein, good for microscopic examination and culture and sensitivity. The casts may have deteriorated and bacteria may affect true glucose reading.

Random specimen
It is the most common type and most convenient sample. It is good for observing physical characteristics, chemical analysis and identification of casts, crystals and cells.

Second-voided
The first morning specimen is discarded and second specimen is collected. Formed elements remain intact.

Post-prandial
It is collected after meal (usually after 2 hours). It is good for glucose and protein estimation. Urine sugar testing now has limited diagnostic or prognostic value.

Timed specimen
It is a combination of all voiding over a length of time. Two-hour specimen is good for urobilinogen and 24-hour specimen is good for quantitative urinary components estimation. Timed urine specimens are collected in dynamic function tests (see WATER DEPRIVATION TEST on page 364).

Foley catheter
Disinfect a portion of the catheter with alcohol, puncturing the tubing directly with a sterile syringe and needle and aspirate the urine. Place urine in a sterile container, it should never be collected from drainage bag.

Apart from these procedures, urine specimen can also be collected by suprapubic aspiration and cystoscopy.

PHYSICAL EXAMINATION

Volume
Measuring volume of urine in a calibrated cylinder is a very messy procedure, therefore, is not recommended. Better method is to weigh the urine with the container and container without urine. Dividing the net weight of urine with specific gravity gives the volume.

$$\text{Volume of urine} = \frac{\text{weight of urine}}{\text{specific gravity of urine}}$$

Normal 24 hour’s volume depends upon age, fluid intake and weather. In an adult it is 800-

1000 ml with day to night ratio of 2:1 to 3:1. When more than 3000 ml is excreted in 24 hours, it is called **polyuria** and occurs due to excessive fluid intake, chilling of skin, diuretics, during absorption of oedema fluid and exudates, chronic kidney disease, diabetes insipidus, diabetes mellitus, mental disorders, primary hyperaldosteronism and hyperparathyroidism. When less than 500 ml urine is excreted in 24 hours it is called **oliguria**. This occurs in dehydration, renal insufficiency, cardiac and hepatic insufficiency, acute glomerulonephritis, late stages of chronic renal disease, shock and urinary tract obstruction.

Odour

Normal urine smells slightly aromatic. Presence of ketone bodies (diabetic ketoacidosis) gives a fruity odour. Bacterial decomposition of urine causes ammoniacal smell. Maple syrup like odour occurs either in the presence of pus or contamination with faeces. Certain foods like garlic impart their smell to urine and so do medications like menthol. A mousy odour is present in phenylketonuria.

Colour

Normal colour of urine is pale yellow because of presence of uroerythrin, urochrome and porphyrin pigments. The colour varies with specific gravity and may become deep orange in highly concentrated urine. The colour of urine not only changes in certain diseases but also with ingestion of certain foods, food dyes and medications (Table 9.1). There are many drugs that can impart colour to urine. This possibility should be excluded before interpreting the colour change.

Appearance

Freshly voided urine is clear. It may become cloudy on standing because of amorphous phosphates, urates, oxalates, pus, bacteria, fat and chyle.

***p*H**

*p*H of the urine is the measure of hydrogen ion concentration of the urine. Urine *p*H has limited utility alone and is useful only when related to other information. If urine is left to stand, its *p*H is altered as urea changes to ammonia. Therefore, fresh specimen is tested for pH. Urine *p*H is an important screening test in renal diseases, respiratory diseases, certain metabolic disorders, and some specific therapeutic regimes.

Normal *p*H is acidic (5.0 to 6.5) but kidney has the capability of changing it over a wide range (4.6.0-8.0, mean 6.0). Urine sample may show one of the following reactions when tested with blue and red litmus paper:

1. Acid: *p*H <7.0 (blue litmus changes to red).
2. Alkaline: *p*H >7.0 (red litmus changes to blue).
3. Neutral: *p*H 7.0 (No change of colour in both the litmus).
4. Amphoteric (buffered): When both the litmus shows colour change.

Table 9.1: Colours of urine and possible causes.

Colour of urine	Possible cause
Straw to amber	Normal (urochrome)
Orange	Concentrated urine, Furoxon, Rhubarb
Deep yellow	Riboflavin, senna
Riboflavin, senna	Pyridium and amidopyrin drugs
Orange brown	Urobilin
Greenish orange	Bilirubin
Smokey	Red blood cells
Reddish brown	Haemoglobin or uroporphyrins
Brown to black on standing	Melanin or homogentisic acid
Almost colourless	Dilute urine
Reddish orange in alkaline	Rhubarb or senna
Dirty green on standing	Excess indican
Red in alkaline	Phenolphthalein
Green or blue	Methylene blue
Greenish yellow fluorescence	Flavones in some vitamin preparation
Brown or black	Phenols, Aralen
Very pale or greenish yellow	Diseases causing polyuria
Orange	Dehydration
Blue green	Blue diaper syndrome
Red	Haemoglobinuria, Beets in food
Pink	Porphyria and Myoglobinuria
Black	Alkaptonuria
Yellow	Carrots in food, Atbrine, Phenacetin
Different colours depending upon dye used	Food dyes

pH of urine is checked with indicator paper or strips. Strips carry methyl red (red strips) and bromothymol blue (blue strip), provide a *p*H range of 5.0-9.0. Strip is dipped in urine and touching the edge of container drains of excess urine. Colour is compared with colour chart. Highly acid urine is observed in high protein diet, ammonium chloride ingestion, diarrhoea, metabolic or respiratory acidosis, chronic obstructive pulmonary disease (COPO), diabetes mellitus, gout, starvation and sleep. Alkaline *p*H is observed in bacterial decomposition of urine at room temperature, bacterial infection, physiological alkaline tide, vegetarian diet, drugs, renal failure, pyloric obstruction, vomiting and metabolic alkalosis. Alkaline *p*H of the urine is also observed in UTI with urea producing organisms

Specific Gravity

This test has significance in the interpretation of other results. Reference range for urine specific gravity is 1.010-1.025. In early morning specimen it may be 1.020. It is low in kidney

diseases, abnormal anti-diuretic hormone excretion and newborn babies (1.002-1.004), and high in dehydration, fever and vomiting. Many contrast agents excreted in urine interfere with conventional specific gravity measurements. Urine should be collected before administration of contrast medium or at a gap of two or more days afterwards. Contrast agents do not distort the colorimetric methods. It may exceed 1.050 if calculated by urinometer

Determination

Specific gravity can be determined by urinometer, refractometer, or by automated equipment. If a urinometer is used for this purpose then the urine is allowed to come to room temperature and is mixed well. Urinometer tube is filled by urine and urinometer is floated in to it. Lower meniscus is read on the scale and is corrected for temperature as most urinometers are calibrated at 20°C. For each change of 3°C, 0.001 is added or subtracted. With each 1% protein in urine the specific gravity increases by 0.003, while for each 1% of glucose it increases by 0.004. In specimens containing these substances, specific gravity should be corrected accordingly. Specific gravity over 1.020 (hyperesthenuria) occurs in decreased intake of fluids, fever, dehydration, and IV albumin administration. Specific gravity less than 1.009 (hypoesthenuria) occurs in increased fluid intake, hypothermia, alkalosis, progressive renal failure and sickle cell anaemia. Specific gravity fixed at 1.010 occurs in chronic renal failure or end stage kidney disease.

CHEMICAL EXAMINATION

PROTEINS

In normal urine protein is undetectable by routine methods. It is an important indicator of renal diseases. It may be used to monitor therapy in renal disease. Protein is found in urine in hypertension, pre-eclamptic toxaemia, renal parenchymal diseases, urinary tract infections, etc. Proteins in urine can be measured qualitatively by heat, turbidimetric and colorimetric methods.

1. **Turbidimetric method**: This can be done by heat (boiling), heat and acetic acid, sulfosalicylic acid test or nitric acid ring test.
   a. **Heat method**: Heat coagulates protein (albumin) much as boiling coagulates egg white. Heat with acetic acid precipitates albumin, globulins and proteoses.
   Procedure: Fill 3/4 of a tube with urine and heat upper part of it while rotating the tube. Turbidity will appear if proteins or phosphates are present. Add 2-3 drops of acetic acid, if turbidity persists it is due to proteins.
   b. **Acid precipitation**: Many chemical agents like sulphosalicylic acid, nitric acid precipitate proteins. Other constituents of urine may also be precipitated with these chemical agents.
   c. **Sulfosalicylic acid test (Kingsbury and Clark)**: Test is based on the principle that proteins are denatured and precipitated by acids.
   Procedure: One ml centrifuged urine is taken in two test tubes. To one tube 3 ml of 3% sulfosalicylic acid is added, while the other tube with urine only acts as a blank. Both the tubes are allowed to stand for 10 min. The tubes are compared for turbidity and also with commercially available standards (Kingsbury Clark standards). Normal urine contains protein up to 7.5 mg/100 ml and does not produce turbidity. The results are reported as trace, or + to +++ roughly corresponding to protein concentration of 20 mg, 30 mg, 50 mg and 75 mg/100 ml respectively. Turbidity produced by albumin is 4 times that produced by globulins. **False positive** results are obtained with mucous, iodine contrast media, metabolites of tolbutamide, plasma expanders, IV albumin and sulfisoxazole. X-Ray contrast media (false positive) may persist for three days after administration. Alkaline, highly buffered urine gives **false negative** result. Improper test technique may give false positive or negative result.
2. **Colorimetric**: At *p*H 3.0 tetrabromophenol blue is yellow in absence of protein whereas in presence of protein it becomes green to blue colour depending upon amount of protein. Sulfosalicylic acid, citrate buffer nitric acid and tetrabromophenol blue are placed on a test area urine test strip. In another type trichloracetic acid with Exton's reagent (sulfosalicylic acid, sodium sulphate and bromophenol blue) is used. These tests are very sensitive and will detect proteins from 0.05-0.2g/L. The results, therefore, should be confirmed with turbidimetric method. The test is specific for albumin. **False positive** results are common in alkaline urine, highly buffered urine and

hypochlorite. Haemoglobin, globulins, Bence Jones proteins give **false negative** reaction. Improper matching colour blocks, poor lighting, etc. may give false positive or negative results.

GLUCOSE AND REDUCING SUGARS

Principal: Monosaccharide hexoses (Table 9.2) are all reducing sugars producing colour reaction when tested with Benedict's reagent or with clinitest tablets (Ames Division, Miles Laboratories). Naturally occurring **polysaccharides** are long-chain carbohydrates composed of glucose subunits:

- **Glycogen**, found in animal tissue, is a highly branched polysaccharide;
- **Starch**, found in plants, is a mixture of amylose (straight chains) and amylopectin (branched chains).

Table 9.2: Common reducing and non-reducing sugars

	Reducing sugars	Non-reducing sugars
Monosaccharides	Glucose Fructose Galactose	
Diasaccharides	Lactose (galactose+glucose	Sucrose (fructose+glucose)
	Maltose (glucose+glucose)	

Glucose is the most common sugar excreted in urine. The normal adult may excrete up to 130 mg glucose/24 hours. However, there are a number of other reducing sugars and reducing substances, which can be present in urine (Table 9.4). The glucose appears in excess of normal minute amount in urine in diabetes mellitus, renal glycosuria, post gastrectomy, epinephrine excess either from the adrenals or injected for therapeutic purposes, pancreatitis, hyperthyroidism, liver damage, renal tubular disease, heavy meal, and emotional stress.

Benedict's test:

For screening urine for reducing substances a non-specific copper reduction method like *Benedict's test* or the one incorporated in *Clinitest tablets* can be used (Table 9.4).

Principle: Soluble blue cupric ions of $CuSO_4$ in heated, strongly alkaline solution are reduced by urinary reducing agents to yellow-red insoluble cuprous ions of Cu_2O.

Blue Cupric ions ($CuSO_4$)+Reducing sugar→ Cuprous ions (Cu_2O) (Orange to Red)+ Oxidised sugar

Procedure: Take 5 ml of Benedict's reagent in a test tube and heat to exclude false positive test. Add 0.5 ml urine. Boil for another 2 min and cool under running tap water. Look for the colour of precipitate. Interpret the result according to Table 9.3. Method is sensitive to glucose concentration as low as 0.2%. The test is positive for all reducing substances given in Table 9.4 and also with salicylates, chloral hydrate, formalin, Vitamin C, drug metabolites e.g., nalidixic acid, first generation cephalosporins etc.

Table 9.3: Interpretation of Benedict's test

	Result	Colour	Amount
	Negative	Blue	0%
	+	Green	0.5%
	++	Yellow	1.0%
	+++	Orange	1.5%
	++++	Red	2.0%

Table 9.4: Reducing substances in urine that may give a positive reaction with Clinitest tablet/Benedict's test

Reducing substance	Comment
Glucose Glucoronates	Common
Lactose	Common in pregnancy
Galactose Fructose Pentose Homogentisic acid	Rare
Urate Creatinine	Weakly positive at high concentrations

Enzymatic test

This is specific for glucose and is now available on dipsticks. The test is based on the principle that glucose is converted to gluconic acid and H_2O_2 by glucose oxidase in presence of oxygen. This H_2O_2 reacts with orthotoluidine in presence of peroxidase to produce coloured compounds. In this case oxidised orthotoluidine (blue) + water. All reagents are provided on a dipstick pad. This test is sensitive to as low as 0.1% glucose. No normal urine constituent gives false negative or positive result. Presence of bleach and peroxides (used for cleaning containers) may give **false positive** results. Very high doses of Vitamin C and homogentisic acid may give **false negativ**e results. For using sticks precautions given by manufacturers must be followed. A positive Benedict's test and negative enzymatic glucose test may indicate presence of non-glucose reducing substances such as galactose, pentose or lactose.

Galactose: It indicates galactosaemia, which is an inborn error of carbohydrate metabolism. (page 356) Galactose-1-phosphate-uridyl-transferase converts galactose to glucose-1-phosphate in liver. Its deficiency results in accumulation of galactose due to metabolic block. It is not a common condition and occurs in infancy. The infant cannot properly metabolise lactose or galactose and develop cataracts, liver damage and possibly mental retardation. The final identification of galactose in urine can be done by chromatography (see THIN LAYER

CHROMATOGRAPHY on page 40 and on page 375).

Pentose: It indicates pentosuria, which is an inborn error of metabolism. Pentose-L-xylulose is excreted in the urine. Pentosuria can also occur after the ingestion of raw plums or cherries. It is checked by the bial-orcinol test.

Lactose: This sugar may be found in the urine in late pregnancy, lactation or in patients on extremely high milk diets. Lactose intolerance with lactosuria is a rare metabolic disease.

BILE PIGMENTS (BILIRUBIN)

This test is required for screening, diagnosis and monitoring of liver, biliary and haemolytic diseases. Normally, urine bilirubin is less than 0.03 mg/dl and is undetectable by routine tests. It may appear before other signs are noticeable. Bilirubin is found in urine in cirrhosis of the liver, viral hepatitis, carcinoma head of pancreas and other bile duct obstructions, and haemolysis. Bilirubin in urine can be detected by:

- **Foam test**: Shaking urine specimen and observing colour of foam (green, yellow or brown). It is insensitive and is now obsolete.
- **Dye dilution test**: Methylene blue is added until urine turns blue. It is also insensitive and thus obsolete (detects bilirubin ≥2 mg/dl).
- **Fouchet's test**: Barium Chloride precipitates phosphates and concentrates bile pigments which are tested for by the oxidation reaction. The pigment is oxidised to green biliverdin by Fouchet's reagent prepared by mixing stock trichloracetic acid solution (page 50) equivalent to 25 g, 10 ml 10% aqueous ferric chloride and making the volume to 100 ml with distilled water.
  Procedure: Add 1g barium chloride to 10 ml urine in a test tube, mix thoroughly and filter. Spread the filter paper. When partly dry, put a few drops of Fouchet's reagent. Green (biliverdin) or blue (cholecyanin) colours indicate a positive reaction. The sensitivity varies from 0.005 to 1.0 mg/dl. False positive test may be obtained with salicylates but the colour produced is purple and Pyridium like substances (phenazopyridine) give red colour. Pigments of urine also obscure positive reaction.
- **Diazotisation Test**: In this test a stabilised diazo compound reacts with bilirubin to form a blue colour.
  Procedure: To 10 ml urine add and mix 4 ml of 10% barium chloride. Mark upper level of fluid with marker. Centrifuge and decant completely. Add distilled water to the mark, mix, centrifuge, and decant completely. Add 0.5 ml Diazo reagent, 2 ml absolute alcohol and 0.3 ml 6% hydrated disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$). Mix and centrifuge. Presence of bilirubin is indicated by supernatant fluid becoming red due to azobilirubin. It is sensitive to 0.05 mg/dl and is specific. For preparation of Diazo reagent see on page 327. This test is also available on dipsticks in which stable diazotised salts are used. The test is very sensitive and can detect bilirubin as low as 0.2 mg/100 ml. The test should be performed on fresh urine only. Very large amounts of phenothiazine (chlorpromazine) metabolites give false positive result. If pyridium like substances are present, they give red colour.

BILE SALTS

Hay's test is employed based on the principle that bile salts lower surface tension because of that light powdered sulphur sinks to the bottom.

Procedure: Take 5 ml urine in a test tube and sprinkle on its surface a bit of finely powdered sulphur granules. If it sinks, bile salts are present in the urine. False positive may be reported because of sinking of heavy impurities in sulphur powder.

BLOOD

This can be haematuria, haemoglobinuria, or myoglobinuria (Table 9.5). In haematuria intact red blood cells (RBC) are present in the urine (lesion of kidney or post-renal bleeding, cancer in urinary tract, urinary tract infections etc.). In haemoglobinuria **free** haemoglobin is present in the urine. It occurs in intravascular haemolysis (transfusion reactions, autoimmune haemolytic anaemia, etc.), severe burn and allergic reactions. In myoglobinuria myoglobin (muscle pigment) is present in the urine. It may result from trauma (crush injury, bullet, beating) unaccustomed exercise (football, swimming) and muscle diseases. Haematuria can be detected by examining urine deposit under the microscope. However, there are certain chemical tests available which can determine the presence of RBCs, haemoglobin and myoglobin.

- **Guaiacum reaction**
  Boil 5 ml urine, cool and add 2 drops of tincture guaiacum. Shake and make a layer with ozonic ether. Blue ring will indicate blood (low sensitivity).
- **Reduced phenolphthalein test**
  Take 3 ml of reduced phenolphthalein and add 10 drop of H_2O_2 and 3 ml urine. Shake well. Pink colour indicates blood.

Table 9.5: Differentiation between haematuria, haemoglobinuria and myoglobinuria

Condition/test	Microscopy	Chemical test	Saturated Ammonium sulphate precipitation test
Haematuria	Positive	Positive	Not done
Haemoglobinuria	Negative	Positive	Positive
Myoglobinuria	Negative	Positive	Negative

- **Pyramidone ring test**
  Take 2-3 ml urine and add few drops of acetic acid. Add slowly equal volume of 5% pyramidone and 5-6 drops of H_2O_2. A mauve colour ring indicates blood.

- **Benzidine test**
  Take 2 ml urine and add few drops of acetic acid and knifepoint of benzidine powder. (see also TEST FOR BLOOD IN FAECES on page 93). Mix to make saturated solution and add few drops of H_2O_2. A blue colour indicates blood (highly sensitive).

- **Commercial dipstick test**
  These dipsticks work on following principle:

Cumerine hydrogen peroxide+o-Toluidine (Haemoglobin+ O_2 + 6-Methoxyquinoline)→Oxidised o-Toluidine (Green-Blue)

The test is most sensitive for free haemoglobin (0.15 mg/dl) or 5-15 intact RBCs/µl).

NITRITE

Normal urine contains nitrates and many bacteria convert nitrates to nitrites. Detection of Nitrites in urine indicates urinary tract infection or contamination. Early morning specimen gives best result. The test is done by commercial dipstick working on following principle:

Urinary nitrate+ Bacterial reductase→Urinary nitrite
Nitrite+*p*-Arsanillic Acid→Diazonium compound
Diazonium+naphthylamine→Diazonium complex (Pink)

KETONE BODIES

Ketone bodies are breakdown products of fat metabolism. These are exhaled from lungs and stimulate respiration. These consist of acetoacetic acid, β-hydroxybutyric acid and acetone. These are normally present in concentrations of up to 125 mg in 24 hours urine but cannot be detected by routine testing. In ketosis the quantity may be as high as 50g in 24 hours. These may appear in urine in starvation, uncontrolled diabetes mellitus, prolonged vomiting, severe diarrhoea in children, low carbohydrate diet, high fat diet and toxaemia of pregnancy. Ketone bodies are tested by the following:

- **Rothera tube test**
  Alkaline nitroprusside with acetoacetic acid or acetone gives purple colour but no colour with β-Hydroxybutyric acid. The test is also available in commercial dipsticks and tablets. This test is more sensitive to acetoacetic acid and detects as low as 10 mg of acetoacetic acid/100 ml of urine. The test must be performed on fresh urine before acetoacetic acid breaks down to acetone. Rothera test is not standardised and vary in sensitivity depending on the amount of reagents and their order of addition. Large amounts of phenylketones or L-dopa metabolites may cause **false positive** results.

- **Gerhardt's test (Ferric chloride test)**
  This test detects acetoacetic acid and is simple to perform. A few drops of 10% aqueous ferric chloride solution are added to 1 ml urine. Appearance of red colour indicates present of acetoacetic acid. The test detects 0.5-1.0 mmol/L (5-10 mg/dl) of acetoacetic acid in urine. Gerhardt test will show **false positive** results with salicylates, PAS and antipyrines (these will not be destroyed by boiling whereas acetoacetic acid evaporates).

UROBILINOGEN

It is a pigment produced by bacterial decomposition of bilirubin in intestine, from where it is reabsorbed and appears in urine. Trace amounts are normally present. Increased amounts indicate increased production of bilirubin. The test is required to detect haemolysis and in the differential diagnosis of jaundice. The test must be performed on freshly voided urine as urobilinogen is converted to urobilin on exposure to light and air. Urobilinogen may be increased in toxic hepatitis, glandular fever, haemolytic anaemia and carcinoma head of pancreas. Following methods are used for its determination:

- **Spectroscopic examination**: Acidify urine with HCl and examine with spectroscope. Absorption band at junction of green and blue indicates presence of urobilinogen (see section on SPECTROSCOPYon page 45).
- **Bogomolow's test**: To 10 ml urine add 0.5 ml of 20% copper sulphate and 4 ml chloroform. Mix by inversion. Urobilin turns chloroform layer pink or yellow.
- **Ehrlich's benzaldehyde test**: Colourless urobilinogen is converted to coloured compound with Ehrlich reagent prepared by mixing 100 ml concentrated HCl with 100 ml distilled water to which 4 g of *p*-dimethylaminobenzaldehyde is dissolved.
  Procedure: To 10 ml urine add 1 ml of the

reagent, mix and let stand for 10 min. Observe colour by looking down into the tube held over a white surface. Cherry red colour indicates a positive result. If no colour is produced observe the tubes again after heating and if again there is no colour, urobilinogen is absent. The test, if positive needs to be repeated on diluted urine until only a faint pink colour is produced. The result is reported as increased (positive reaction in ≥1/16 dilution), present but not increased (positive in dilution <1/16) and absent (no reaction even after heating). **False positive** reactions may be seen. Urobilinogen is decreased or is absent in newborns when there is complete obstruction of the common bile duct, starvation, intrahepatic cholestasis and intestinal sterilisation. It is increased in haemolysis with or without jaundice.

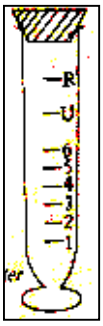
BENCE JONES PROTEINS (BJP)

These are light chains of globins with a molecular weight of 45,000. They are found in 40% cases of multiple myeloma and other lymphoproliferative disorders with monoclonal dysglobulinaemia. Since they are small molecules, they are easily cleared from plasma by kidneys and excreted in the urine. These proteins give positive sulfosalicylic acid test for proteins but only a weak positive or no reaction with dipstick.

Heat precipitation test
Principle: BJP precipitate at about 60°C and re-dissolve near 100°C. When the urine is cooled these reappear between 85°C and 60°C.
Procedure: Centrifuge fresh urine and take 10 ml of clear urine in a test tube. Check *p*H and adjust to 5.0 with 25% acetic acid. Place a thermometer in the test tube and heat slowly in a water bath. If BJP are present, clouding will begin at 40°C and precipitation will be complete at 60°C. Now take out the thermometer and boil the urine in the test tube. The precipitate will disappear. Replace thermometer in test tube and cool. Precipitate reappears and then fades to disappear at temperature below 40°C. This test should be confirmed by electrophoresis of concentrated urine.

QUANTITATIVE TEST FOR PROTEINS

- **Esbach's test**
  The test is based on protein precipitation by picric acid[1]. Esbach's reagent consists of 1% picric acid. The instrument used is called Esbach's albuminometer, a graduated tube placed in wooden cover.



  Procedure: Dilute filtered urine sample with distilled water to a specific gravity between 1.006-1.008 and amount of water used should be noted. If alkaline, the reaction should be changed to acid with 1-2 drops of 22% acetic acid. Fill Esbach's tube to mark U. Add Esbach's reagent to mark R. Mix by gentle inversion about 12 times. Replace in the case, stopper and leave for 24 hours. Read the height of white protein precipitate in grams per litre. Correct for any dilution. This, however, is not an accurate method of protein estimation.

- **Pyrogallol red dye test**
  Principle: The pyrogallol red molybdate complexed with protein at *p*H 2.5 gives violet coloured compound measured at 600 nm, which is proportional to the concentration of proteins. The method is sensitive to the mg range suitable for both urine and CSF protein measurement. The method can also be used to measure **microalbuminuria** (See microalbuminuria on page 325.). This method has also been automated.
  Pyragallol red dye: Dissolve 10 mg of disodium molybdate, 5.9 g of succinic acid, 134 mg of sodium oxalate and 430 mg of sodium benzoate in about 800 ml of distilled water. To this add 25 mg of pyrogallol red dye and mix well till it is completely dissolved. Make up to 1L. Store in an amber bottle. Stable at 2-8°C for 3 months.
  Procedure: To 3 ml regent add 50 µl sample, standard and control. Mix all tubes well. Leave at 25-35°C for 15 minutes. Set the spectrophotometer to zero using blank at 600 nm (red filter) and measure the absorbance of standards, test and control.

PHENYLKETONURIA

In this disease there is increased concentration of phenylalanine in blood and CSF due to deficiency of hepatic phenylalanine hydroxylase. Phenylketones are excreted in urine and can be detected with Ferric chloride test.
Procedure: To 5 ml fresh urine, add 3-5 drops of 10% aqueous ferric chloride. Greyish green to blue green colour appears within 90 seconds and disappears after sometime.

[1] Picric acid is always kept hydrated under water. Dehydrated picric acid can explode. The saturated solution of picric acid (stock solution) is appropriately diluted to make reagents.

PORPHOBILINOGEN

Watson-Schwartz test
It is based on the principle that Ehrlich reagent turns porphobilinogen into a red coloured compound, which differs in solubility from red compound produced by urobilinogen and indole. Fisher's modified Ehrlich reagent is used (20.7g *p*-dimethylaminobenzaldehyde dissolved in 150 ml concentrated HCl added to 100 ml distilled water).
Procedure: Mix 2.5 ml fresh urine with 2.5 ml Ehrlich reagent, shake for 30 seconds (immediate red colour is due to porpho-bilinogen). Add 5 ml saturated sodium acetate and mix well. Adjust *p*H to 5.5 with more sodium acetate if required. If colour appears after addition of sodium acetate, it is most likely due to urobilinogen. If colour appears, add 5 ml chloroform to reaction mixture, shake well and allow to stand. Porphobilinogen will remain in aqueous upper layer while urobilinogen is extracted in the lower chloroform layer. To confirm, separate upper aqueous layer and mix it with equal volume of *n*-butanol. Allow to separate. If the colour is due to porphobilinogen it will separate with aqueous lower layer (see also The PORPHYRIAS on page 344).

PORPHYRIN

To 5 ml of fresh urine add 0.75 ml glacial acetic acid and 1.5 ml amyl alcohol. Mix well and centrifuge to separate the layers. Examine under ultraviolet light. Salmon pink fluorescence in upper layer of amyl alcohol indicates porphyrin in excess of normal.

CALCIUM

Calcium in urine is screened by Sulkowitch test. Calcium is precipitated as calcium oxalate by oxalic acid reagent prepared by dissolving 2.5 g oxalic acid, 2.5 g ammonium oxalate and 5 ml glacial acetic acid in 1L distilled water. A 24 hours urine sample is required. Mix 5 ml urine with 5 ml reagent and observe for turbidity. No precipitate is due to normal calcium, whereas high calcium gives a heavy precipitate (Table 9.6).

Table 9.6: Interpretation of Sulkowitch test

No precipitate	Serum calcium <7.5 mg/100 ml
Fine precipitate	Serum calcium 7.5-11.5 mg/100 ml
Heavy precipitate	Serum calcium ≥11.5 mg/100 ml

CHLORIDE

Chloride is tested by Fontana's test in which chloride is precipitated with silver nitrate, excess of which then produces reddish precipitate of silver chromate with potassium chromate. A 20% solution of potassium chromate and 2.9% solution of silver nitrate are required.
Procedure: In a test tube place 10 drops of urine and one drop of potassium chromate. Add silver nitrate drop by drop until permanent distinct red brown colour appears. Same dropper should be used for urine and reagents. Number of drops required to produce the colour change is equal to number of gram of sodium chloride per litre of urine. Normal urine requires 6-12 drops.

MICROSCOPIC EXAMINATION

Microscopic examination is an essential component of urinalyses. Following examination procedures are carried out.

Light microscopy
It is carried out to see ova or parasites (Trichomonas, Schistosoma, Echinococcus, Filaria larvae), RBCs, leukocytes, casts, epithelial cell and crystals. Bacteria, yeast, cylindroids, spermatozoa, mucous, fat and artefacts can also be seen.
Preparation of deposit: Centrifuge 10-15 ml well mixed urine at 1000 rpm for 3 min. Invert the tube to pour off the supernatant. Mix the sediment with the small amount of urine left in the tube. Pour a drop on a clean glass slide and put a cover slip. Examine under subdued light, first scanning whole area with the low power objective and then with the high power objective. Amorphous deposit may cover important structures; therefore, they should be removed by adding a small drop of 10% acetic acid, which dissolves the deposit. Too much acid should be avoided, as it will dissolve the casts. The features to be noted under low power are casts, spermatozoa, mucous threads, yeast, fat droplets and ova of parasites. Casts are reported as number per low power field and rest of the elements as few, moderate or many.

Leucocytes
Normal urine from males does not contain more than 1 leucocyte per high power field (HPF), while from females it contains 1-5 cells/HPF. These are usually polymorphs and may show amoeboid movements in fresh specimen. Increased number (pyuria) indicates inflammation and occurs almost in all renal diseases (Figure 9.1 and Figure 9.2). Leukocyte casts are present, if infection is of renal origin. Some causes of pyuria (pus in the urine) are acute or chronic pyelonephritis, acute or chronic cystitis, renal tuberculosis and bladder trauma. Leukocytes are rapidly lysed in hypotonic alkaline urine. Approximately 50% may be lost in 2 to 3 hours at room temperature. Therefore,

urine should be examined as early as possible after collection.

Erythrocytes

These appear as highly refractile, round, yellowish structures (Figure 9.3). Normal urine from males does not contain any RBC except if the specimen is collected by catheterisation. Urine from female may show a few RBCs from vaginal contamination or many during menstruation. With these two exceptions presence of RBCs in the urine (haematuria) is a significant finding. Increased number of red cells may originate in any part of the urinary system. In case of renal origin, urine will have RBCs, red cell casts, proteinuria and dysmorphic RBCs. Some causes of renal haematuria are glomerulonephritis, lupus nephritis, calculus, tumour, trauma, acute infection, etc. If origin is lower urinary tract (acute and chronic infection, calculus, tumour of urinary bladder and stricture of urethra) urine will have red cells but no cast and no protein.

Casts

Casts are cylindrical structures with parallel sides and blunt rounded ends that quickly dissolve in alkaline urine. These are formed in tubules and may even be present when tests for albumin are negative. They are translucent, colourless gels. Their size and shape depends on tubules where they were formed. They indicate widespread kidney disease. These often occur intermittently and may not be seen in all specimens. They are basically composed of mucus protein, called **Tamm-Horsfall** protein, forming a matrix in which are incorporated other elements depending upon the type of cast. Casts are increased in acidity, urinary stasis, increased plasma proteins, and high solute concentration. A morphological variant is called **cylindroid**. Its structure is similar to cast but its shape is different. It tapers at one end and may thin down into a thread at that end. Another morphological variant is a very broad cast, which is formed in collecting tubules. It is also called renal failure cast. Different types of casts seen in urine are:

- Hyaline casts in which no other elements are mixed in the basic structure (Figure 9.4).
- Red cell casts when RBCs are trapped in the matrix (Figure 9.5).
- Pus casts when pus cells are present in the cast (Figure 9.6 and Figure 9.7).
- Epithelial casts contain epithelial cells (Figure 9.8).
- Fine granular casts, when fine amorphous granules are present in the cast (Figure 9.9 and Figure 9.10).
- Coarse granular casts when the granules are coarse.
- Fatty casts contain fat droplets in matrix or are formed when the contents undergo fatty degeneration (Figure 9.11).
- Bile casts contain bilirubin in matrix.
- Haemoglobin casts are brown and are formed either due to presence of haemoglobin in the cast or due to degeneration of a red cell cast (Figure 9.12).
- Waxy casts are formed in amyloid disease. They are structure-less and colourless like hyaline casts but are more transparent.

Epithelial Cells

Squamous epithelial cells are normally present in small numbers. In females, very large number indicates vaginal contamination. Tubular epithelial cells appear in renal disease. These resemble leucocytes but have a prominent nucleolus in a centrally located nucleus (Figure 9.13). These cells may contain bilirubin or haemosiderin (Figure 9.14).

Amorphous Deposits

Amorphous (fine granular) urates are seen in acid urine while amorphous phosphates are common in alkaline urine (Figure 9.15).

Crystals

These are not seen in fresh warm urine but form after sometime. Except for cystine, uric acid, leucine and tyrosine crystals, they have little significance. The type of crystals seen depend upon the *p*H of urine. Alkaline urine contains triple phosphate (ammonium-magnesium phosphate) (Figure 9.16), calcium carbonate and ammonium biurate crystals. Acid urine may contain calcium oxalate (Figure 9.17), uric acid (Figure 9.18), cystine (Figure 9.19, Figure 9.20 and Figure 9.21), tyrosine (Figure 9.22) and leucine (Figure 9.23) crystals. Other crystals include cholesterol (Figure 9.24) and drugs like sulpha, which crystallise in urine (Figure 9.25 and Figure 9.26).

Miscellaneous

Besides these, many other things are seen in the urinary sediment. These include ova of *Schistosoma haematobium* (page 93), malignant cells, bacteria, yeast cell (Figure 9.27), trichomonas, filaria (page 114), mucous threads (Figure 9.28) etc.

Dark ground illumination is required when organisms like Leptospira are expected.

Staining of urine sediment: Following stains are used for urine microscopy:

- Methylene blue
- Sternheimer-Malbin stain

Polymorphs are orange purple, Hyaline cast

pale blue or colourless, RBCs lavender; cellular cast pink to purple; trichomonas light blue; nuclei of bladder epithelial cells blue and vaginal epithelial cell nuclei purple). Stains for Fat Cells include Sudan III, Sudan IV and Oil Red O. Acid Fast stains include Ziehl-Neelsen and Kinyoun stains.

AUTOMATED INSTRUMENTATION

There are many automated equipments (like Clinitek 100, urilux) being used for routine urine examination. These equipments eliminate variability of visual interpretation, are more convenient and accurate, allow computer interfacing and reduce need for re-testing (see AUTOMATED URINE STRIP READER on page 58).

MICROSCOPIC TEST FOR FAT

This test is based on staining of fat with Sudan III.

Procedure: Mix a few drops of 36% acetic acid on a slide with few drops from the top surface of centrifuged urine. Add several drops of saturated solution of Sudan III in 95% ethanol and heat to boiling for few seconds. Examine under the microscope. Fat appears as deep orange globules that become spiked on cooling. To see neutral fats use 95% ethanol in place of acetic acid. Neutral fat appear as yellow to pale orange globules (Figure 9.29).

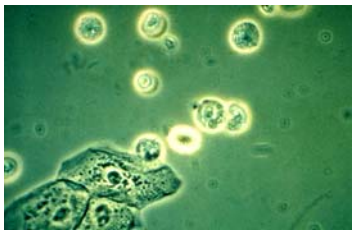


Figure 9.1: WBCs and epithelial cells

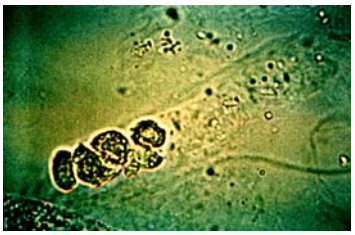


Figure 9.6: Pus cell (WBC) cast

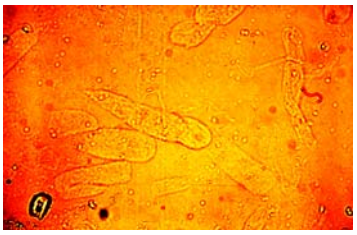


Figure 9.11: Hyaline and waxy casts

Figure 9.2: White Blood Cells

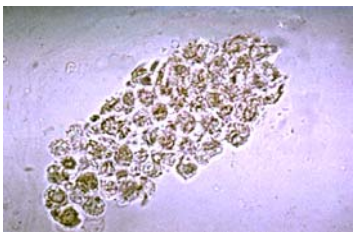


Figure 9.7: WBC cast

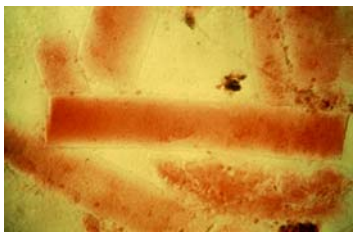


Figure 9.12: Haemoglobin cast

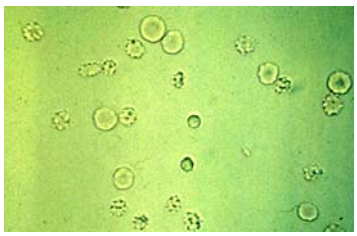


Figure 9.3: Red blood cells

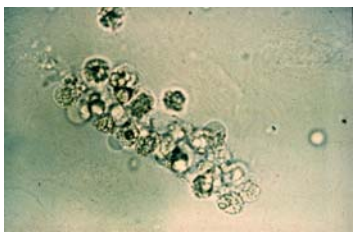


Figure 9.8: Epithelial cast

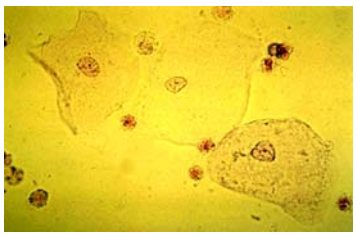


Figure 9.13: Epithelial cells

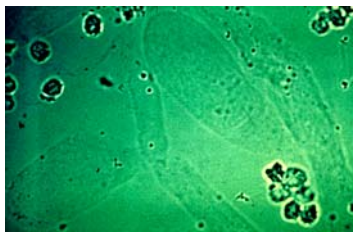


Figure 9.4: Hyaline cast with pus cells

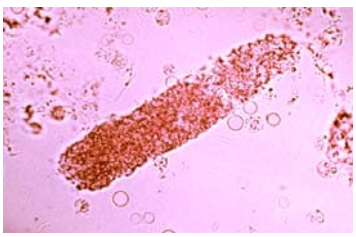


Figure 9.9: Granular casts

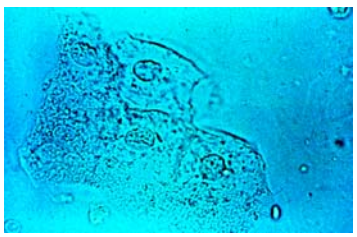


Figure 9.14: Epithelial cells

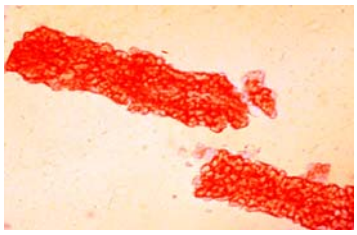


Figure 9.5: Red cell cast

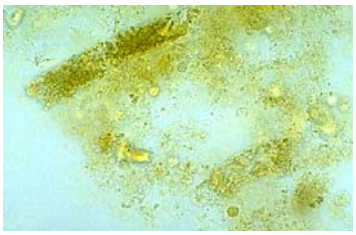


Figure 9.10: Granular casts

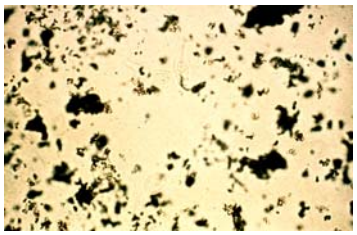


Figure 9.15: Amorphous phosphates/ urates

Figures 9.1-9.15 Urine deposit

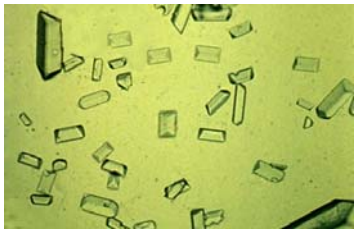


Figure 9.16: Triple phosphate crystals

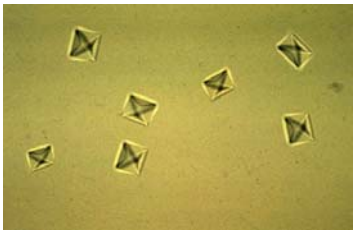


Figure 9.17: Calcium oxalate crystals

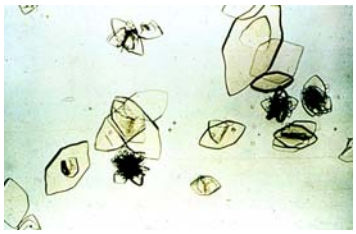


Figure 9.18: Uric acid crystals

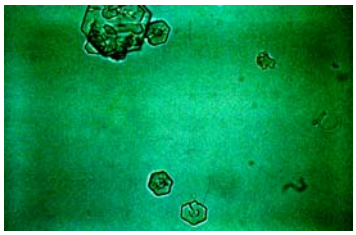


Figure 9.19: Cystine crystals

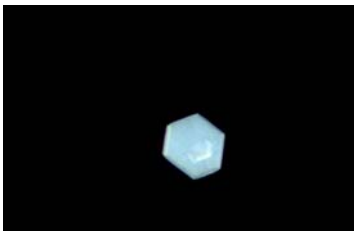


Figure 9.20: Cystine crystal (phase contrast)

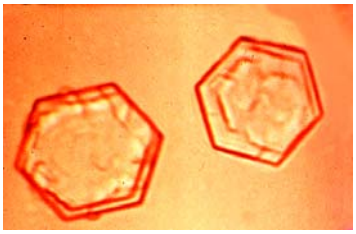


Figure 9.21: Cystine crystal

Figure 9.22: Tyrosine crystals

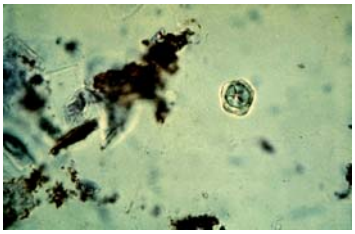


Figure 9.23: Leucine crystals

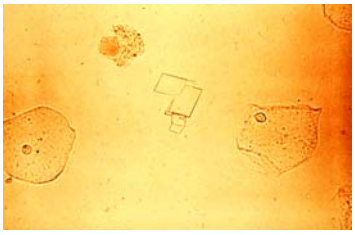


Figure 9.24: Cholesterol crystals

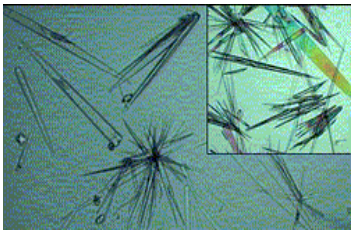


Figure 9.25: Drug crystals

Figure 9.26: Drug crystals

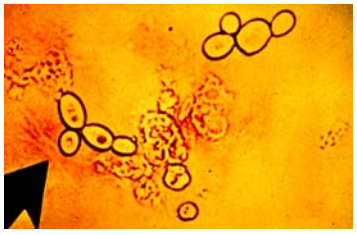


Figure 9.27: Yeast cells

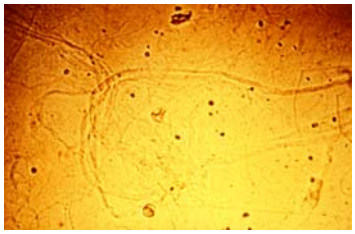


Figure 9.28: Mucous threads

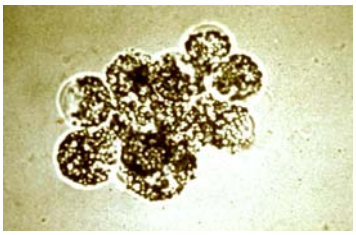


Figure 9.29: Fat bodies

Figures 9.16-9.28 Urine deposit

10. EXAMINATION OF FAECES

Faeces are mainly composed of remains of ingested food, dead intestinal bacteria (normal flora), and denuded/shredded mucosa. Food undergoes processes of digestion and absorption while it traverses about 20 feet of intestine. The frequency of faeces depends upon the personal habits. The quantity passed in 24 hours depends upon food habits and time taken by it to pass through the intestinal length. In addition faeces also contain substances excreted through bile into intestine. The gut is one of the highest contaminated viscera in the body and bacteria present here also modify the substances present inside the intestine.

COLLECTION OF FAECES

The faeces can be collected in bedpan and care should be taken to prevent the mixing with urine. From bedpan a suitable portion is transferred to an appropriate container such as a waxed cardboard box, empty tin with a lid, a light plastic box or to a specially designed glass jar for faeces collection with a spoon attached to the stopper. The specimen should at least be 4 ml (4 cm^3) in quantity. The collection of sufficient quantity is necessary in order to permit detection of parasites in low concentration and to prevent rapid drying of faeces. The care should be taken that the actual abnormal part (mucus and blood) is collected and sent to the laboratory immediately, preferably within one hour. It is important especially when vegetative form of amoebae is to be seen. If a number of specimens are received at the same time, liquid faeces and those containing mucus or blood are examined first (see also COLLECTION OF SPECIMEN on page 68).

PHYSICAL EXAMINATION

Colour
Normal colour of faeces is due to the presence of stercobilinogen produced by bacteria through decomposition of bilirubin. On exposure to air it is converted to brown stercobilin. As breast fed infants have no bacteria in their intestine so stercobilinogen is not produced and the colour of faeces remain yellow. In diarrhoea the movement of intestine is so rapid that bacteria do not have time to decompose bilirubin and green faeces may be passed. Colour of faeces depends upon various factors. The concentration of bile pigments gives a greenish colour to faeces particularly in diarrhoea of infants (starvation faeces). On the other hand obstruction to the flow of bile into the intestine, gives rise to pale, tan or clay coloured faeces. Chlorophyll rich foods produce green faeces. Bleeding into upper gut gives rise to black faeces due to altered blood. If bleeding is in the lower part of the intestine then the colour of faeces is red. In addition, oral iron ingestion results in black faeces. Various drugs will change the colour of the faeces accordingly.

Odour
Normal odour is because of indole and skatole. It varies with *p*H and is dependent on bacterial fermentation and putrefaction. Faeces are particularly offensive in amoebic dysentery.

Consistency
Normally faeces are formed or semi-formed. The faeces can be liquid, semi-liquid, solid, semi-solid and foamy. Solid or hard faeces are passed in constipation and loose faeces in diarrhoea. Diarrhoeal faeces mixed with mucus and blood is seen in amoebic dysentery, carcinoma of large bowel and typhoid. Loose faeces mixed with pus and mucus occurs in bacillary dysentery, regional enteritis and ulcerative colitis. Paste like and frothy loose faeces are seen in sprue, pancreatic insufficiency and other malabsorption syndromes. Watery faeces (rice water faeces) are seen in cholera.

Parasites
Intact parasites like *Ascaris lumbricoides* and *Enterobius vermicularis* or segments of *Taenia saginata* may be seen with naked eye. Even smaller worms and scoleces can be seen when faeces are liquefied with water and strained through a wide mesh sieve and restrained through a medium mesh sieve.

Reaction or *p*H
Normal *p*H of faeces is either neutral or weakly alkaline. In general, on mixed and meat diet the reaction tend to be alkaline and in predominantly carbohydrate or fat-rich diet, acid. Carbohydrate breakdown changes the *p*H to acid (as in amoebic dysentery) and protein break down changes it to alkaline (as in bacillary dysentery).

In cases of lactose intolerance in infants because of excessive fermentation of lactose the faeces tends to be highly acidic.

MICROSCOPIC EXAMINATION

DIRECT WET PREPARATION

A small portion of freshly passed faeces is examined by making a thin suspension in a drop of normal saline and drop of Lugol's iodine on a glass slide. This is covered with a cover glass. Faeces should be selected both from the exterior as well as the central portion of the faecal mass. Faecal matter selected for examination should contain blood and mucus, in case of blood stained faeces. Microscopically one will see, food residues (digested and undigested muscle fibres, fat globule and fatty acid crystals, starch granules and cellulose residues), cells (RBCs, WBCs and epithelial), crystals (triple phosphate, calcium oxalate, cholesterol and Charcot Leyden crystals), ova (*Ascaris lumbricoides, Enterobius vermicularis, Ancylostoma duodenale* etc.), parasites or their cysts and mucus and foreign bodies (hair, wool etc.). This method also demonstrates motile amoebae, which contain ingested RBC and show purposeful, unidirectional movement by throwing out pseudopodia. Ova and cysts can be seen by moving the objective of the microscope up and down and keeping the light subdued. Addition of a drop of Lugol's iodine from the edge of the cover slip provides a good contrast and stains some inclusions of protozoan cysts like glycogen mass. Normal structures should not be confused with abnormal findings like ova and cysts. These include hair, vegetable fibres, starch cells, yeasts and spores, muscle fibres, fat globules and pollen grains.

CONCENTRATION TECHNIQUES

These methods are used when ova or parasites are not found in direct saline preparation but their presence is highly suspected or symptoms persist. Ova of certain parasites are scanty e.g., *Schistosoma, Taenia* etc. so may require concentration methods for their demonstration. These methods are:

- **Formalin ether sedimentation**
  Concentration techniques using formalin not only kill the parasites but also fix them preserving their morphology, therefore, these are considered the best.
  Procedure: Emulsify about 2 ml of faeces in 3 ml of saline in a 15 ml conical centrifuge tube; add saline to 15 ml mark. Centrifuge at 1500 RPM for one min. Decant the supernatant and re-suspend deposit in another 15 ml saline. Repeat until clean sediment remains. Mix with 10 ml 10% formalin and allow to stand for 5 min. Add 3 ml ether, stopper the tube and shake vigorously. Remove the stopper and centrifuge at 1500 RPM for 2 min. Four layers from bottom upwards are will be; sediment containing parasites, formalin, faecal debris and upper most layer of ether. Free the faecal debris from walls and remove top three layers. Re-suspend deposit, prepare saline and iodine wet films and examine under the microscope.

- **Sodium chloride floatation technique**
  The faeces are mixed with saturated solution of sodium chloride. The eggs are lighter in weight, so these float to the surface.
  Procedure: Place about 2 ml of faeces in an empty clean small bottle or tube. Quarter-fill the bottle with saturated solution of sodium chloride (SSS). Mix faeces with the help of an applicator and fill the bottle to the top with SSS. Place a cover slip over the mouth of the bottle so that it touches the liquid without having air bubbles in between. Remove the cover slip; a drop of liquid should remain on it. Place the cover slip on a slide and examine under the microscope.

- **Zinc sulphate floatation procedure**
  Parasitic cysts and some Helminth eggs will rise to the surface of a liquid having high specific gravity (zinc sulphate, specific gravity 1.180), due to their buoyant properties in that solution. The solution of zinc sulphate can be prepared by adding 330 g of dry crystals of zinc sulphate in 670 ml distilled water.
  Procedure: Prepare a faecal suspension of ¼ to ½ teaspoon in 10-15 ml of water. Filter this material through two layers of gauze into a small tube. Fill the tube with tap water to within 2-3 mm of the top and centrifuge for 1 min at 500 X g. Decant the supernatant fluid, fill the tube with water, and re-suspend the sediment by stirring with an applicator stick. Centrifuge for 1 min at 500xg. Decant the water, add 2-3 ml zinc sulphate solution, re-suspend the sediment, and fill the tube with zinc sulphate solution to within 0.5 cm of the top. Centrifuge for 1-2 min at 500xg, allow the tube to come to stop without interference or vibration. Without removing the tube from the centrifuge, touch the

surface of the film of suspension with a wire loop, parallel to the surface. Add the material in the loop to a slide containing a drop of dilute iodine or saline. (The slide should be examined as soon as possible, because high specific gravity will distort the ova).

Morphology of various protozoa, cysts, and ova found in stools is summarised below. Details are discussed in the section on Parasitology (on page 107).

PROTOZOA

Entamoeba histolytica

Vegetative form is free living unicellular organism, active, motile, with the help of pseudopodia and contains ingested RBCs (motility is called as amoeboid movement). Size varies from 12-35 µm. While moving, it is elongated and changes shape but round when stationary or static. It has unidirectional movement. The ectoplasm is transparent and endoplasm is finely granular and greyish or yellowish green in colour. The cytoplasm contains 1-20 RBCs. The nucleus in motile amoebae is not visible but in iodine preparation nucleus with clear membrane and central dense karyosome is visible.

The cysts are sharply outlined, refractile, round structures, 12-15 µm in diameter. They contain 1-4 nuclei. The nuclear membrane is thin, regular and circular and a small central karyosome is easily visible in iodine stained preparation. Cytoplasm in iodine preparation is yellowish grey and granular. It contains a glycogen mass and chromatoid bodies (oblong, rounded at ends; in only immature cyst) (Figure 10.1). The following characteristics are valuable in identification of *E.histolytica*:

Unstained trophozoites: Progressive motility, hyaline pseudopodia, no ingested bacteria and invisible nuclei are suggestive. Ingestion of red cells is diagnostic.

Stained trophozoites: Clear differentiation of ectoplasm and endoplasm, no ingested bacteria are suggestive, whereas fine, uniform granules of peripheral chromatin and small central karyosome in nucleus, ingested red cell and average size over 12 µm is diagnostic.

Unstained cyst: Four nuclei and rod like chromatid bodies are suggestive.

Stained cysts: Maximum of four nuclei having both karyosome and peripheral chromatin and diameter over 10 µm is suggestive, whereas typical nuclear structure, chromatid bars with rounded or squared ends and diameter over 10 µm is diagnostic.

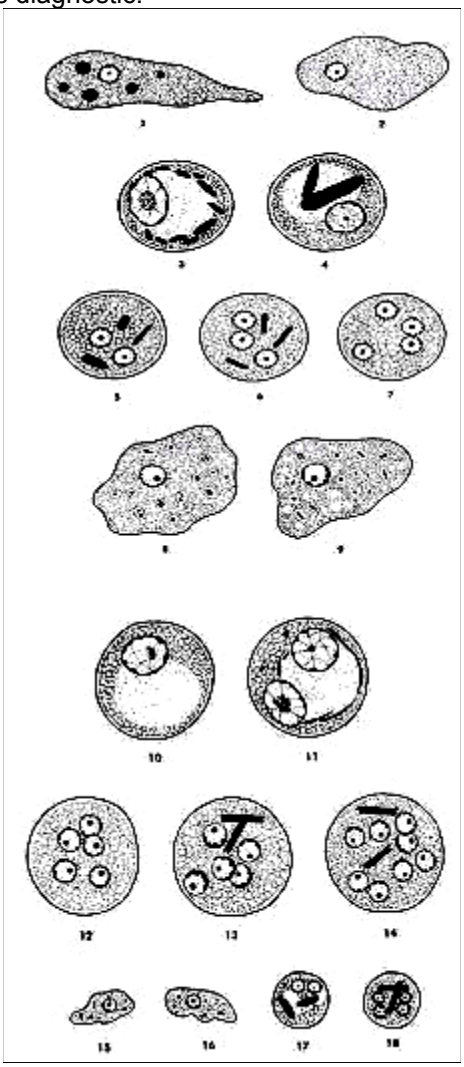
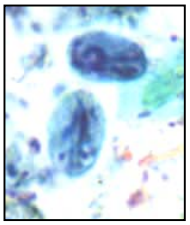


Figure 10.1: Protozoa in faeces. 1,2, Trophozoites of *Entamoeba histolytica*. 3, 4, early cysts of *Entamoeba histolytica*. 5-7, Cysts of *Entamoeba histolytica*. 8,9, Trophozoites *of Entamoeba coli*. 10,11, Early cysts of *Entamoeba coli*. 12-14, Cysts of *Entamoeba coli*. 15,16, Trophozoites of *Entamoeba hartmanni*. 17, 18, Cysts of Entamoeba *hartmann*i

Giardia lamblia

Vegetative form is kite or pear shaped (front view) or spoon shaped (side view), flagellated, motile organism (classically like a falling leaf). They are 10-18 µm in size. There are two nuclei and four pairs of flagella. It shows spinning or rapid jerky movements. Two large oval nuclei are faintly visible.

Cysts are small (8-12 µm), oval and refractile, containing 2-4 nuclei usually at one end with small faintly coloured central karyosome. Two curved longitudinal axostyles



are seen in the centre. The cytoplasm is shrunk away from the wall. The shell is double walled and thick. Following characteristics are important for identification of *Giardia lamblia* trophozoites and cysts:

Unstained trophozoites: Progressive, **falling leaf** motility; pear shaped body with attenuated posterior end is suggestive.

Stained trophozoites: Nuclei in the area of a sucking disk; the two median bodies, posterior to the sucking disk and typical arrangement of axonemes are diagnostic.

Unstained cysts: Ovoid shape of the body and numerous refractile threads in cytoplasm are suggestive.

Stained cysts: Four nuclei, four median bodies and jumble of axonemes are diagnostic.

HELMINTHS

Taenia saginata* and *Taenia solium

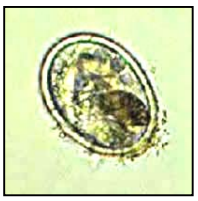
The eggs of both tapeworms are similar. Eggs are spheroid, yellow to brown in colour and 30-40 µm in diameter (embryophore). The thick, radially striated shell is dark yellowish brown in colour covering light yellowish grey material. Inside is a narrow clear space, lined by a thin membrane, in which lies a granular mass, the hexacanth embryo, with 3 pairs of refractile, lancet shaped hooklets (oncosphere).

Ascaris lumbricoides

There are four types of eggs of Ascaris:

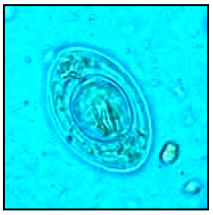
1. **Fertilised ova with double shell**: They are yellow brown with thick shell having an uneven rough, brown, albuminous outer coat and thick, smooth, transparent inner shell. These measure 50x70 µm and contain unsegmented fertilized ovum as single, round, granular, central mass with clear crescentric spaces on either pole.

2. **Unfertilised ova with double shell**: These are elongated, 50x90 µm in size. The two shells are indistinct. Inner shell is thin and filled with coarse granular or globular cytoplasm, outer shell is brown, and puffy with rather jagged lumps. The eggs are full of large, round, very refractile granules.



3. **Semi-decorticated fertilised eggs**: These have single inner shell only. It is thick and colourless and contains a single round colourless granular central mass.
4. **Semi-decorticated unfertilised eggs**: These have single inner shell only. It is thin colourless with double lines and contains large round colourless refractile granules.

Hymenolepis nana



Ovum is nearly spherical, 45 µm in diameter. It has two distinct walls; external membrane is thin and internal membrane is often thicker at poles with 4-8 hair like filaments coming out from both poles. Some granules occupy the space between the two membranes. It contains rounded mass of a gelatinous substance with three pairs of refractile hooklets arranged in fan shape and often some well-defined granules in the centre (Hexacanth embryo).

Enterobius vermicularis

Ovum is asymmetrically ovoid with one side flattened. The size is 20x50 µm. It is transparent and colourless. There is a thin, double line shell, with a coiled larva inside or a small, granular mass in the shape of an irregular oval figure.

Strongyloides stercoralis

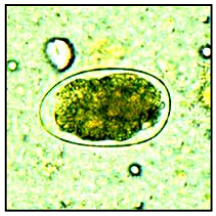
Rhabditiform larvae are demonstrated by concentration technique. Larva is 200-300 µm and is un-sheathed. Digestive tube has a swelling at one end (oesophagus) and another (anal pore) at other end. The tail is moderately tapered. The genital primordium is a rounded, clear space near the middle. The eggs are usually not found in faeces because they hatch before evacuation but liquid faeces may contain them. They are very similar to that of *Ancylostoma duodenale* but are slightly smaller (50 µm).

Trichuris trichura

Ova are characteristically barrel shaped and measure 50 µm in length. These are rounded and transparent with plugs at both ends. These have fairly thick and smooth shell with two layers. The shell is orange in colour while contents are yellow. They contain a uniform granular mass

(un-segmented ovum).

***Ancylostoma duodenale* (Hookworm)**



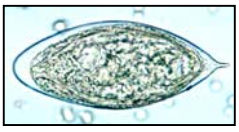
Ovum is oval with rounded slightly flattened poles, colourless with very thin shell that appears as black line. It measures 40x60 µm in size. It contains segmented embryo of 4 to 16 cells stage that is pale grey but turns dark brown with iodine solution. The contents varies according to the degree of maturity:

1. Fresh faeces have grey granular, clear cell.
2. Few hours old faeces will have a uniform mass of many small grey granular cells.
3. 12-48 hours faeces will have small larvae in place of cells.

Schistosoma haematobium

Ova are usually found in urine but sometimes in faeces also. They measure 50x150 µm, oval, elongated and dilated in the middle. The ovum is grey or pale yellow in colour with smooth and very thin shell. It has short terminal spine and contains fully developed ciliated embryo (miracidium) surrounded by a membrane.

Schistosoma japonicum



Ova are pale yellow or colourless, almost rounded, measuring 70x80 µm. The spine is lateral and small, seen with difficulty. It contains fully developed broad ciliated embryo (miracidium)

Schistosoma mansoni

Ova are pale yellow, oval with a lateral (near the round pole), large, triangular spine. The egg measures 50x150 µm and it has smooth very thin shell. It contains a fully developed ciliated embryo (miracidium), surrounded by a membrane. Calcified egg is usually smaller and black with a less distinct spine.

TEST FOR BLOOD IN FAECES

Blood in faeces can be detected by:

Benzidine Test[1]

This test detects microscopic blood in faeces. More than 10 ml of blood will give a black colour to the faeces, whereas, less then 10 ml (occult) blood from gastrointestinal tract will be detected by this test. Peroxidase in the haem of haemoglobin liberates oxygen from hydrogen peroxide that oxidises benzidine in acidic medium and changes it to blue coloured compound. **False positive** test is given by meat. The patient is asked to avoid meat one day before the examination. He should not take any iron-containing compound and brush his teeth.

Procedure: Make a suspension of faeces in 10 ml saline and boil to inactivate the normally present oxidising enzymes in faeces. Make 2 ml of saturated solution of benzidine in glacial acetic acid in other tube. Add 2 ml of H_2O_2 and check whether blue or green colour develops. If so discard the reagents. Add faecal suspension drop by drop to the solution of benzidine and H_2O_2 till there is change of colour.

Appearance of deep blue colour indicates presence of blood.

Orthotoluidine Test

Orthotoluidine is converted to blue coloured compound by blood. Two percent sodium perborate solution in water and 2% orthotoluidine solution in glacial acetic acid are mixed in equal volume just before use. Add 6 drops to a smear of faeces on a filter paper. Blue colour indicates presence of occult blood test. These tests also form basis of commercially available strips.

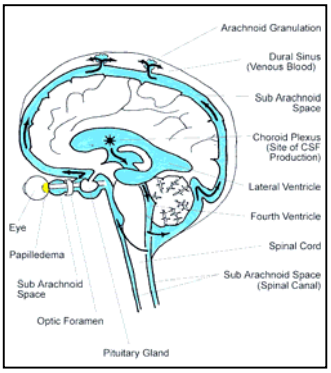
Guaiacum Test

This test can also be used for testing blood in stools and is available commercially. For details see Guaiacum reaction on page 81.

[1] Benzidine is a carcinogen therefore one should be very careful while using it

11. EXAMINATION OF CEREBROSPINAL FLUID (CSF)

Cerebrospinal fluid (CSF) is contained in a cavity that surrounds the brain in the skull and the spinal column. It nourishes the tissues of the central nervous system and helps to protect the brain and spinal cord from injury. Choroid plexuses present in ventricles of the brain secrete it continuously at a rate of 500 ml/day. From here it circulates the subarachnoid space of both brain and spinal cord and is absorbed into blood of dural venous sinuses by arachnoid villi. CSF is composed of substances present in plasma but its composition differs, as it is not formed by simple filtration. Entry of many substances into CSF is controlled by so called **Blood Brain Barrier**, which allows free entry of some substances into CSF but inhibits the entry of others. This barrier is however, deranged in inflammation. Therefore, changes in composition of CSF may occur not only in diseases of brain and spinal cord but also in metabolic diseases like diabetes etc. Main function of CSF is protective. It provides a fluid cushion for brain to protect it from injuries that may otherwise occur due to sudden movements inside bony cavity. It also maintains the volume of the brain inside cranial cavity and provides some nutrition. It also cleans neuronal tissue of wastes. The normal volume of the CSF is 100-150 ml.



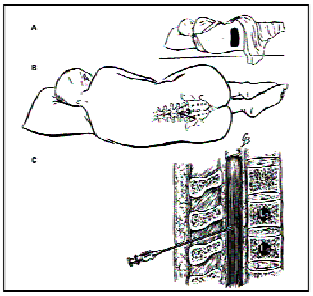
NORMAL CSF

Normal CSF is a colourless, clear, watery fluid and no coagulum or pellicle is formed when it is allowed to stand undisturbed in a refrigerator. It contains only 1-5 cells/mm^3 and these are lymphocytes. Chemical composition is as follows:

Proteins: 0.2-0.45 g/L (20-45 mg/dl). Higher the level of collection of CSF lower is the protein. Therefore, in ventricular fluid these are only 50-150 mg/L. In neonates protein concentration may be as high as 1.7 g/L.

Glucose: It is 2.5-4.5 mmol/L (45-80 mg/dl) and the value is usually 2/3 of the blood glucose level at any time. In diabetes or continuous intravenous glucose infusion the value may be high. It is better that a sample for blood glucose shall also be collected simultaneously to make the interpretation easy.

Chlorides: 118-127 mmol/L. The estimation of chlorides is of some value in tuberculous meningitis and heat stroke.



In addition, CSF contains other plasma crystalloids too but these are not determined in routine examination.

SAMPLE COLLECTION AND STORAGE

CSF is normally collected from sub-arachnoid space of spinal cord at lumber level by puncture with a long needle. A physician in the ward under strict aseptic conditions performs the procedure. Specimen shall be collected in 2-4 ml quantities in 3-4 sterile screw capped bottles that are serially numbered and must be sent to the laboratory immediately. In case CSF is to be cultured for *M. tuberculosis* then at least 5 ml sample is needed. CSF shall be tested as soon as it arrives in the laboratory. CSF in the first bottle is sometimes contaminated with blood and should be kept aside. Fluid from second bottle is used for routine tests while fluid from third bottle is used for bacterial culture etc. If tuberculous meningitis is suspected, 4th bottle is kept in refrigerator undisturbed to see whether a pellicle or coagulum forms. Otherwise CSF must never be refrigerated (if for bacterial culture as it kills *H.influenzae*) and should be kept at 37°C.

ROUTINE EXAMINATION

Appearance

First of all note the colour of CSF in all three bottles. If blood is visible it should be noted whether it is present in all bottles equally or it is present in first bottle and then disappears. The amount of blood shall also be noted. If there is gross contamination of CSF with blood in all bottles then the chemical values will not be true. If no blood is seen, then note the colour. A yellowish colour (Xanthochromia) is commonly

seen in subarachnoid haemorrhage persisting for several weeks, in neonatal period, brain tissue destruction and sometimes in long standing jaundice. Psudomonal meningitis may be associated with bright green CSF. Note the translucency or turbidity. If the number of WBCs is high in the CSF, then the fluid becomes turbid. In such cases cell count can be omitted with main emphasis on Gram stain and culture. Finally check if there is clot or pellicle formation in the CSF. It indicates increased fibrinogen in CSF that is a sign of inflammation.

Cell Count

The CSF may contain WBC in varying quantity in certain diseases. The cell count should be carried out as soon as possible after collection of specimen, since the cells are rapidly lysed. Table 11.1 depicts the WBC counts in different CSF samples.

Table 11.1: WBC count in various conditions

Conditions	WBC count /mm^3	Predominant cell type
Normal adult CSF	0-25	Lymphocytes
Normal neonatal CSF	<30	Neutrophils
Tuberculous meningitis	100-500	Neutrophils
Viral meningitis	10-500	Lymphocytes

If CSF is clear then the cells can be counted by charging a Neubauer counting chamber with well-mixed, uncentrifuged, undiluted fluid. Cells in all the nine WBC squares shall be counted (see DETERMINATION OF TOTAL LEUCOCYTE COUNT (TLC) on page 253). The number of cells counted is approximately the number of cells per mm^3 of CSF. If the count is expected to be high then CSF has to be diluted for cell counting. Diluting fluid for CSF is prepared by dissolving 200 mg crystal violet in 100 ml of 10% acetic acid. Method for counting and calculation is same as for counting of WBC in peripheral blood. In case there is gross contamination of CSF with blood, blood derived leucocytes would be present in CSF, therefore, the count is to be corrected. For this purpose perform RBC and WBC count in both CSF and peripheral blood.

If:

Blood RBC count = RBC(B)
CSF RBC count = RBC(F)
Blood WBC count = WBC(B)
CSF WBC count = WBC(F)

then

$$\text{True CSF cell count} = \frac{\text{WBC(F)} - \text{WBC(B)} \times \text{RBC(F)}}{\text{RBC(B)}}$$

The finding of >1 WBC/1000 RBCs will suggest the presence of meningitis.

Microscopic examination

If the CSF does not contain numerous cells (<200x10^9/L), centrifuge 2-4 ml CSF in a conical test tube, preferably, at a slow speed for 5-10 min. Save most of the supernatant in a clean test tube for chemical analysis. Re-suspend the sediment in a drop of remaining CSF. Prepare at least three smears on glass slides and dry these in air.

Stain one smear with Leishman stain (for type of WBC), one with Gram method (for presence and type of bacteria) and the third with Ziehl-Neelsen method of staining (for acid-fast bacilli).

Special preparations can be made if required, e.g., India ink preparation or Nigrosine staining if *Cryptococcus* is suspected or direct wet preparation for trypanosomes and *Neglaria* spp.

ESTIMATION OF PROTEINS

Increase in protein is the commonest abnormality of CSF. Proteins should always be estimated quantitatively. Various methods are available for this purpose. Easiest is turbidimetric method using proteinometer. *Proteinometer* is a set of standard tubes showing turbidity of known amount of proteins in CSF.

Mestrezat's Diaphenometric Procedure

Place 2 ml CSF in small test tube (sugar tube) and add to it 0.3 ml 30% trichloracetic acid. Shake well and place in a water bath at 100°C for 2 min. Set aside for 20 min or longer. Then compare turbidity with standard tubes.

Sulfosalicylic acid test

Take 3 ml of 3% sulfosalicylic acid in a tube and add 1 ml of supernatant clear CSF in it. Cloudiness of the test is compared with that of standard tube.

Biuret Method

Principle: CSF proteins can be estimated calorimetrically by using Biuret or Kingsbury method.

Reagents: Trichloracetic acid 10%, Sodium hydroxide 15%, Copper sulphate 5%

Procedure: To 2 ml CSF add 2 ml 10% trichloracetic acid, mix well and allow to stand for 5 min. Centrifuge at high speed and discard the supernatant. Mark this tube containing precipitate as **test**. Take another test tube and mark it **blank**. To both tubes add 1 ml 15% NaOH. Shake the "test" tube to dissolve the precipitate. Add 0.5 ml 5% Copper sulphate and 4 ml distilled water. Mix thoroughly and centrifuge at high speed. Transfer the supernatant to corresponding clean tubes marked. Read absorbance of the "test" against "blank" in a colorimeter at 550 nm. Read the

quantity of proteins from calibration curve.

Preparation of standard Curve: Take pooled serum and determine its protein content by standard method for serum. Dilute with normal saline so as to obtain a concentration of 2 g/L. Set up series of tubes as shown in Table 11.2. Treat each tube as "test" making only one blank and add 2 ml of 10% trichloracetic acid. Shake well and let stand for 5 min. Note absorbance. Plot these on a linear graph paper against concentration (see PREPARATION OF CALIBRATION CURVE on page 47 for details).

Dye Binding method

There are certain dyes that bind with protein to give colour complexes. These have been used for measuring small amounts of proteins in body fluid such as CSF. Initially **Coomassie Brilliant Blue** (CBB) was used for this purpose. Although it was very sensitive and specific, but it had the disadvantage of staining all the glassware. The method has now been replaced with other dyes.

Pyrogallol red method: See page 325.

Doubt is often expressed about values of protein estimation when CSF contains red cells and therefore added plasma protein. Calculation shows that 1400 red cell per ml (mm^3) of CSF fluid correspond approximately to 1 mg of added proteins per 100 ml of CSF. The adjustment can be made as follow:

RBC = 30000 per 1 mm^2

Protein = 220 mg per 100 ml

$$\text{Corrected protein} = 220 - \frac{30000}{1400} = 200 \text{ mg per } 100 \text{ ml (approx)}$$

Estimation of Globulins

This test is quite useful and in absence of contamination by blood, a positive reaction is always pathological. Normal CSF contains traces of globulin (about 3 mg/100 ml), but not sufficient to react positively. The test is almost always positive, when total protein exceeds 100 mg/100 ml. The following test is performed:

Pandy's test: A qualitative Pandy's Test is sufficient for routine purposes.

Pandy's Reagent: Dissolve 10g phenol in 150 ml distilled water. Reagent should be clear and colourless.

Procedure: Take 2 ml reagent in a test tube and add 2-3 drops of CSF. Examine the solution after each drop. Opalescence will appear in the reagent that vary in intensity. Only a slight opalescence is significant and indicates increased globulins. A coat of white precipitate forms around drop of CSF when it travels through reagent.

Nonne-Apelt reaction

This test will also determine the globulin in CSF. In this test 1 ml CSF is mixed with 1 ml saturated ammonium sulphate solution and shaken well. Keep the mixture aside for 3-4 min. Normal CSF will remain clear whereas, increased globulins produce opalescence, turbidity or precipitate. (Normal CSF may give slight opalescent).

ESTIMATION OF GLUCOSE

Glucose in the CSF is rapidly destroyed once the fluid is collected, it is, therefore, important to carry out glucose estimation as soon as possible. If there is likely to be a delay, the CSF should be preserved in fluoride oxalate. Any method of blood glucose estimation can be used. Since the amount of glucose in CSF is less than that in blood and may further be reduced due to disease therefore volume of CSF used in the test should be twice that of blood used in the same procedure. For details of method see blood glucose estimation in section of chemical pathology on page 265.

Table 11.2: Preparation of calibration curve for CSF proteins.

Tube	1	2	3	4	5	6	7	8	9
(CSF protein g/L)	0	0.2	0.4	0.6	0.8	1.0	1.2	1.6	2.0
Diluted serum (ml)	0	0.1	0.2	0.3	0.4	0.5	0.6	0.8	1.0
Saline (ml)	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.2	0

Table 11.3: CSF Findings in Some Diseases

Disease	Appearance	Cell Count l	Cell type	Protein g/L	Glucs mmol/ L	Cl' mmol/L
Normal	Clear Colourless	0.5 x109/	Lymphocyte	0.2-0.4	2.5-4.4	112-127
Choriomening itis	Clear	++ Lymphocyte	+	N	N	
Purulent Meningitis	Turbid	+++	Polymorphs	+++	0 –2.5	N
Tuberculous meningitis	Opalescent	++	Lymphocytes	++	1-2.5	85-112
Encephalitis	Clear	N to ↑	Lymphocytes	+	N	N
Brain abscess	Turbid	+	Polymorphs	+ to ++	N to↓	N to ↓
Syphilis	Clear	N to ↑	Lymphocytes	N to ++	N	N to ↓
Tumours	Xanthochromia	N to ↑	Lymphocytes	+++	N	N
Subarachnoid haemorrhage	Bloody or Xanthochromic	N to ↑	RBC	N to +++	N	N
Disseminated Sclerosis	Clear	0-1 x109/	Lymphocytes	0.2-0.4	N	N

ESTIMATION OF CHLORIDES

Readings above 760 mg/dl are most commonly encountered in renal inefficiency and below 700 mg/dl in meningitis. Although it is not usually performed for CSF but it may become useful in diagnosis of tuberculosis meningitis. They are also valuable in cerebral abscess and in other complications of infections in ear and nose.

Reagents: Potassium chromate 5%, Silver nitrate 0.5814%

Procedure: Take 1 ml CSF in a clean test tube and add 2 drops of 5% potassium chromate and

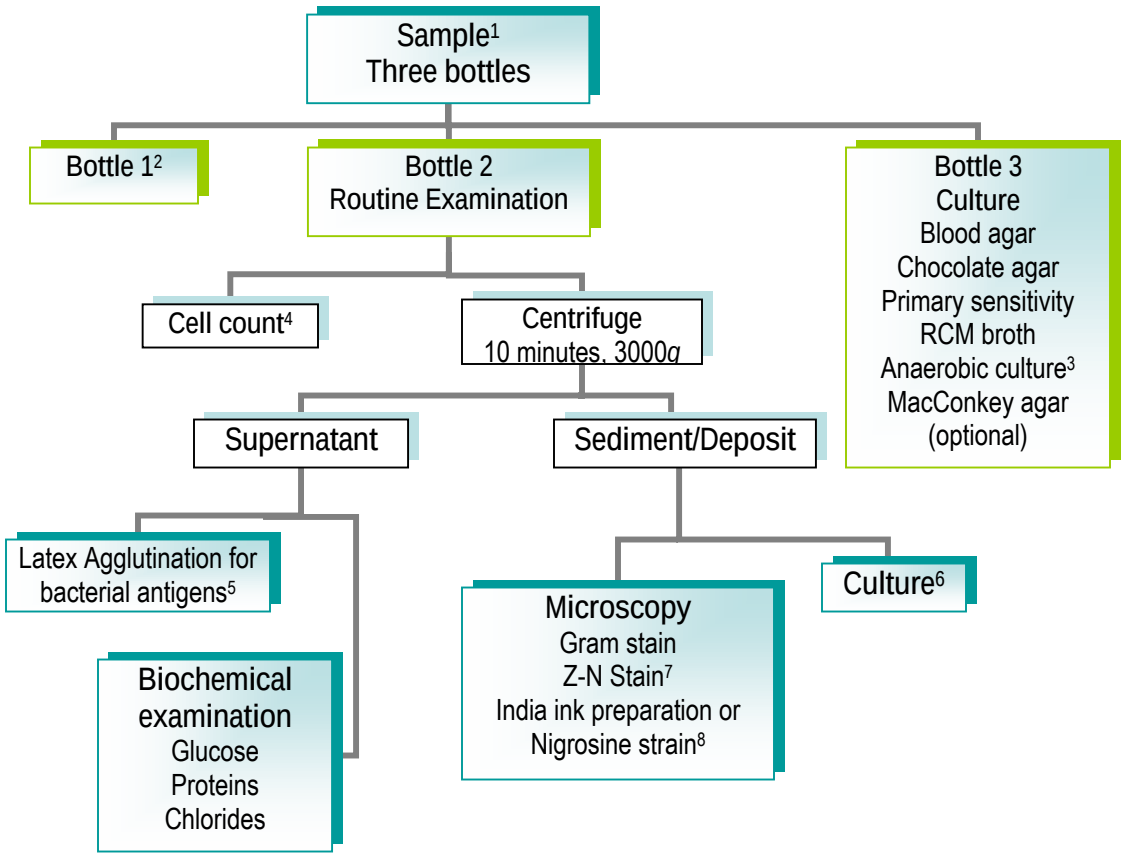
mix. Add silver nitrate with a measuring pipette drop by drop mixing constantly, till permanent yellow to brown colour appears. Note the quantity of silver nitrate used.

Calculation: Quantity of silver nitrate in ml required to produce colour change x 85.5=mmol/L Chloride (as NaCl).

CSF CULTURE

Findings of routine examination indicative of infection make the culture mandatory. Whether culture for *Mycobacterium tuberculosis* and procedures for viral diseases are required will depend upon findings of routine examination and clinician's suspicion. CSF in 3rd bottle is used for these. Methods are discussed in section on Microbiology (page 157).

Figure 11.1: Flowchart for the procedure of CSF examination



1. If less CSF is available, most of CSF examination can be carried out with some limitations. Microbiological analysis/procedures should be done first.
2. Usually contaminated with blood, cell count can be made even with some minor traumatic tap by making dilutions.
3. When brain abscess is present possibly due to anaerobic infection.
4. Uncentrifuged, undiluted sample.
5. Subject to availability of appropriate reagents.
6. If less CSF is available, i.e., no bottle 3, deposit can be used for culture purposes.
7. In suspected tuberculous meningitis.
8. In suspected *Cryptococcus neoformans* meningitis.

12. EXAMINATION OF ASPIRATION FLUIDS

A number of fluids, other than CSF, are received in the laboratory for routine examination. These include:

- Ascitic (peritoneal) fluid
- Pleural fluid
- Pericardial fluid
- Synovial fluid
- Hydrocoele fluid
- Aspirates from cysts, etc.

PLEURAL AND PERICARDIAL FLUIDS

Main purpose of testing is to ascertain their transudative or exudative nature (Table 12.1) and to find a causative organism if an infective process is indicated. Scheme of examination is almost the same as for CSF except that determination of specific gravity is important in these fluids while determination of chloride can be omitted. The most reliable tests for differentiating between a transudate and an exudate is the simultaneous analysis of pleural fluid and serum for total protein and lactic dehydrogenase levels. A transudate is an effusion in which the ratio of serous fluid total protein to serum protein is less than 0.5, while corresponding LD ratio is less than 0.6. If the fluid is labelled as transudate, no other tests are required but if it is exudate then Gram staining, cultures and counterimmuno-electrophoresis is indicated. Cytologic examination and biopsy may be indicated in a case of suspected malignancy (Table 12.2).

SPECIMEN COLLECTION

Medical officer collects specimens in the ward under aseptic conditions. Fluid is collected in 3-4 sterile containers as for CSF. It is good to take a separate specimen in EDTA for cell count.

ROUTINE EXAMINATION

1. **Appearance**: Note the amount, colour and transparency. Normal fluid is straw-coloured and clear without coagulum or pellicle.
2. **Specific gravity**: Determine specific gravity either by refractometer or by using copper sulphate solutions of known specific gravity (see also Specific Gravity on page 78). Normal specific gravity is less than 1.016.
3. **Cell count**: Procedure used is same as for CSF (page 95). Normally these fluids contain 0-8 cells per mm^3 and these are lymphocytes and mesothelial cells.
4. **Preparation of smears** for staining is exactly as for CSF.

Table 12.1: Differences between Transudate and Exudate

	Transudate	Exudate
Appearance	Clear	Cloudy or turbid
Colour	Watery or straw	Turbid to purulent or bloody
Specific gravity	<1.016	≥1.016
Cell count	<1X10^9/L Lymphocytes and mesothelial cells	>1X10^9/L Neutrophils early but mononuclear cells later
RBC	Absent	Often present
Clot formation	None	Usual
Glucose	Same as serum	Same as serum or reduced (>50% of serum level)
Total proteins	<20 g/L (<50% serum level)	≥20 g/L
Rivalta Test	Negative or faint	Positive
LD	<60% of serum activity	>60% of serum activity.
Fluid total protein to serum total protein ratio	<0.5	>0.5
Fluid LD to serum LD ratio	<0.6	>0.6

5. **Estimation of proteins**: method is same as for CSF. However, as protein content of these fluids is higher than that of CSF these should be diluted before protein estimation. Dilution depends upon specific gravity. If specific gravity is high, then further dilution shall be made. Results are then multiplied with dilution factor accordingly.
6. **Estimation of Globulins**: A qualitative test is usually performed. Test performed on serous fluids is Rivalta test. Required reagent is prepared by adding one drop of glacial acetic acid to 100 ml of distilled water in a conical flask. To this are dropped 1-2 drops of centrifuged supernatant fluid. Normal fluids do not produce any cloud in reagent. Transudate produces faint cloud but distinct cloud appears if fluid is exudate.
7. **Estimation of glucose** is important. Glucose levels in pleural fluid below 3.5 mmol/L (60 mg/100 ml) or 2.3 mmol/L (40 mg/100 ml) less than the simultaneous plasma glucose level is considered 'decreased'. Decreased value of glucose in exudates may be seen in bacterial infections, especially when the exudate is purulent, rheumatoid arthritis, malignant

pleuritis and tuberculous pleuritis.

8. α**-Amylase**: Pleural effusion may be the first sign of pancreatic disease. α-Amylase activity should be measured in all unexplained effusions. α-Amylase activity is considered elevated, when the level in fluid is 1.5 to 2.0 times the simultaneous serum level. Pleural fluid α-amylase activity may be increased in a variety of conditions, including acute and chronic pancreatitis, pancreatic pseudocyst, oesophageal rupture, and rarely primary or metastatic carcinoma of lung.
9. **Creatine kinase** isoenzyme BB is high in pleural and pericardial fluids in case of adenocarcinoma of prostate gland. This enzyme is also high in adenocarcinoma and anaplastic carcinoma of lung.
10. **The *p*H** of normal pleural fluid is 7.64. *p*H <7.30 is associated with empyema, malignant disorders, collagen disorders, tuberculosis, oesophageal rupture, or haemothorax. A pleural fluid *p*H 7.3-7.4 usually indicates a benign condition. A *p*H of <6.0 is highly suggestive of oesophageal rupture. The *p*H <7.1 of pericardial fluid is associated with connective tissue diseases and bacterial infection. A *p*H of 7.2-7.4 is associated with neoplasms, idiopathic disorders and tuberculosis or uraemic pericarditis. A *p*H >7.4 is associated with post-cardiotomy states and hypothyroidism.
11. **Staining**: If fluid is exudate and infective process is suspected then cultures must be done. Third container, which was set aside, is used for this purpose. Gram and acid fast staining is fundamental to any fluid examination.
12. **Culture**: In fungal disease, appropriate culture is usually necessary.
13. **Agglutination** techniques for identification of certain bacterial antigens (*S. pneumoniae*) can be done on the fluid.
14. **Tumour Markers**: Determination of Tumour markers in pleural fluid is sometimes helpful in diagnosis of certain malignancies. These are done if presence of malignant cells is suspected. The test is positive in cases of adenocarcinoma lung, carcinoma breast and ovary (Figure 12.1).

In addition to tests mentioned above, few additional tests may also be required.

Test for Viscosity

Aspirate fluid in a pipette and then release. If falling drop draws into a band of 5 cm or long the viscosity is normal. If length of band is less than 5 cm viscosity is decreased (page 104.

Test for Mucin (Hyaluronic Acid)

To 5 ml of 1:5 diluted fluid add 0.14 ml 7N acetic acid (408 ml glacial acetic acid in 1 litre distilled water). Stir with glass rod, examine immediately and after 2 hours. A tight ropy mass is termed good. Softer shreddy precipitate is termed; fair and poor precipitate is shreds of mucin in turbid solution. The later two indicate reduced hyaluronic acid content.

Wet Preparation for Crystals and Inclusions

A drop of fluid is placed on a clean slide and covered lightly with cover slip. Preparation is then examined under microscope with condenser lowered down. Needle-like crystals of urates are seen in gouty arthritis. In rheumatoid arthritis small, multiple, dark inclusions, are seen in polymorphs. These are immunoglobulins with RA factor activity.

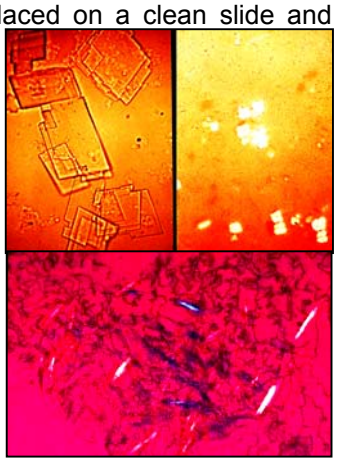


Table 12.2: Work up of pleural effusion

Pleural fluid protein to serum protein ratio	<0.5	No further tests required
Pleural fluid LD[1] to serum LD ratio	<0.6	
Pleural fluid protein to serum protein ratio	>0.5	Gram stain, culture, total WBC and differential counts, cytology, pH, glucose, α-amylase, tumour markers pleural biopsy
Pleural fluid LD to serum LD ratio	>0.6	

PERITONEAL FLUID

The common indications for paracentesis are ascites of unknown origin, suspected intestinal perforation, haemorrhage or infarct, infections like tuberculosis, complications of cirrhosis (spontaneous bacterial peritonitis) and suspected intra-abdominal malignant disorders. To distinguish between ascites caused by liver disease and malignancy, the serum-ascites albumin concentration gradient is more reliable than the ascitic fluid to serum ratio for either total protein or LD. The serum-ascites albumin gradient is greater in transudate (1.6±0.5 g/dl) than exudates (0.6±0.4 g/dl). Peritoneal lavage is useful in evaluating the conditions of patients with blunt trauma. Peritoneal lavage consists of

[1] LD=Lactate dehydrogenase

inserting a peritoneal dialysis catheter into the abdominal cavity through a small midline infra-umbilical incision. The catheter is aspirated and if blood is not observed grossly, 1 litre of Ringer's lactate solution is introduced and immediately retrieved by gravity and interpreted as described in Table 12.3. Table 12.4 depicts various appearances of peritoneal fluid and associated diseases.

Table 12.3: Criteria for diagnosing blunt and penetrating trauma by peritoneal lavage fluid analysis.

Diagnosis	Gross findings	Laboratory findings
Penetrating trauma	Blood in lavage Blood in drain fluid from Foley's catheter or chest tube Evidence of food/foreign particle/bile	RBC count >0.1 million/µl WBCs count >500/µl α-Amylase level >twice that of serum
Blunt injury	None of the above gross findings	RBC count <0.025 million/µl WBC count <100/µl α-Amylase level <serum α-amylase level

Table 12.4: Appearance of peritoneal fluid and associated diseases

Appearance	Disease
Clear, pale-yellow	Cirrhosis
Cloudy, turbid	Bacterial peritonitis, pancreatitis, malignancy
Green	Biliary tract disease, ruptured viscera
Bloody	Trauma, malignancy, pancreatitis, intestinal infarction
Milky	Chylous ascites, trauma, malignancy

MICROSCOPY

Smears are made and stained as usual. A differential cell count with more than 25% neutrophils is considered abnormal. A predominance of neutrophils is suggestive of bacterial infection and an absolute neutrophil count of more than 250/µl is indicative of spontaneous or secondary bacterial peritonitis. A predominance of lymphocytes is seen in congestive cardiac failure, cirrhosis, nephrotic syndrome, chylous effusions, tuberculosis peritonitis and malignant disorder. The fluid is also examined for malignant cells.

CHEMICAL ANALYSIS

Protein

Total protein estimation has little value in differentiating between transudate and exudate. Serum-ascites albumin ratio gives better discrimination (Table 12.1).

Glucose

Simultaneous plasma-fluid glucose ratio of 1.0 or more is suggestive of tuberculosis and abdominal carcinomatosis, ratio of less than 1.0 is seen in cases of cirrhosis or congestive heart failure.

Enzymes

α-Amylase in peritoneal fluid is increased in acute or traumatic pancreatitis or pancreatic pseudocyst however, lipase determination is more reliable in diagnosis of pancreatitis. High level of alkaline phosphatase in the fluid than in the blood is seen in patients with bowel strangulation, intestinal perforation or traumatic haemoperitoneum. Lactate dehydrogenase ratio of ascitic fluid and blood of more than 0.6 is suggestive of abdominal malignancy.

Tumour markers

Carcinoembryonic antigen (CEA) suggests malignancy as a cause of peritoneal fluid accumulation (Figure 12.1).

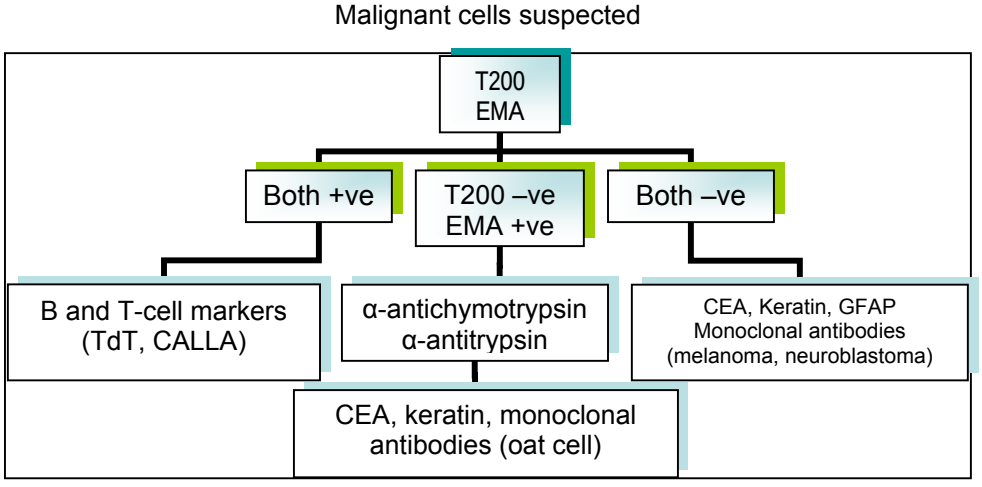


Figure 12.1: Approach for tumour marker interpretation. T200=Panleukocyte antigen, EMA=Epithelial Membrane Antigen, TdT=Terminal deoxynucleotidyl Transferase, CALLA=Common Acute Lymphoblastic Leukaemia Antigen, CEA=Carcinoembryonic Antigen, GFAP=Glial Fibrillary Acidic Protein.

MICROBIOLOGIC EXAMINATION

Culture of peritoneal fluid is often required to identify the microorganisms of tuberculosis peritonitis and spontaneous bacterial peritonitis. This should include aerobic, anaerobic cultures and for organisms requiring CO_2 like *Streptococcus pneumoniae*. Bacterial antigens can be detected by agglutination or counterimmunoelectrophoresis.

SYNOVIAL FLUID

Analysis of synovial fluid plays a major role in the diagnosis of joint diseases. When infective arthritis and crystal-induced synovitis are suspected, examination of the synovial fluid may indicate a definite diagnosis. Through clinical and laboratory examination of the synovial fluid, joint disorders can be divided into five categories (Table 12.5). There are no absolute contraindications to joint aspiration. However, relative contraindications are the presence of local sepsis (cellulitis), bacteraemia, and a congenital or acquired bleeding tendency. Three samples are collected. Five to 10 ml is collected in a sterile tube for microbiological examination; 5 ml is collected in anticoagulant (heparin or EDTA) for microscopic examination; and the third sample is placed in a plain tube and allowed to clot (normal fluid does not clot). If the specimen cannot be examined immediately, fluid should be frozen and stored at -70°C until examined. Routine examination of synovial fluid includes the following:

APPEARANCE

A description of colour and clarity is made.

MICROBIOLOGIC STUDIES

These include examination of stained smears and cultures. The most common organisms are *Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumoniae, Haemophilus influenzae, Neisseria gonorrhoeae* and *Mycobacterium tuberculosis*. If tuberculosis, fungi or anaerobic bacteria are suspected, special handling and culture media are needed. Microbial antigens can be detected by latex or haemagglutination, radioimmunoassay and counterimmunoelectrophoresis.

CELL COUNTS

Theses include total and differential cell counts.

POLARISING LIGHT MICROSCOPY

This is done for crystals including monosodium urate (gout), calcium pyrophosphate dihydrate (pseudo-gout) or crystal deposition disease (CPPD), cholesterol, steroid and hydroxyapatite.

CHEMICAL EXAMINATION

Protein
Normal protein level is one third that of serum, with an average of about 2.0 g/dl. Level higher than 3.0 g/dl suggest an inflammatory or haemorrhagic exudate.

Glucose
Glucose level of synovial fluid is interpreted along with plasma level, which is normally equal to or slightly lower than (within 10 mg/dl) the serum level. For other conditions the variation is depicted in Table 12.6.

Complement level
C3 and C4 levels in the synovial fluid sometimes suggest a disease. In rheumatoid arthritis they are normal or decreased, in SLE they are decreased and in Reiter's disease and gout they are raised above serum level.

Table 12.5: Classification of Arthritides.

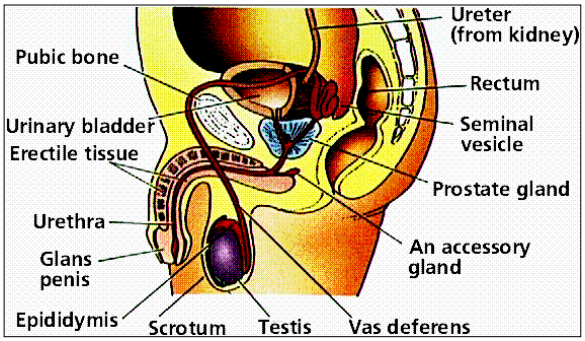
Group I	Group II	Group III	Group IV	Group V
(Noninflammatory)	(Inflammatory)	(Infectious)	(Crystal-induced)	(Haemorrhagic)
Osteoarthrosis	Rheumatoid arthritis	Bacterial	Gout	Traumatic arthritis
Traumatic arthritis	Lupus erythematous	Mycobacterial	CPPD (calcium pyrophosphate dihydrate deposition disease Appetite-associated	Haemophilic arthropathy
Osteochondritis dissecan	Reiter's syndrome	Fungal		Anticoagulation
Osteochondromatosis	Rheumatic fever			Synovial haemangioma
Neuropathic osteo-arthropathy	Ankylosing spondylitis			
Pigmented villo Nodular synovitis	Regional enteritis			
	Ulcerative colitis			
	Psoriasis			

Table 12.6: Synovial fluid findings by disease category.

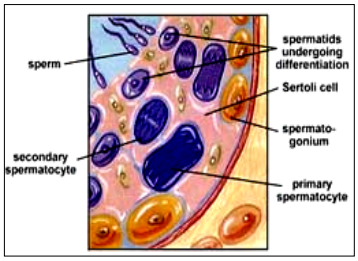
Findings	Normal	Group I Noninflammatory	Group II Inflammatory	Group III Infectious	Group IV Crystal-induced	Group V Haemorrhagic
Appearance		Yellow, clear or slightly cloudy	Yellow or clear turbid or bloody	Yellow, cloudy, or milky	Yellow, green Yellow or turbid	Red-brown or xanthochromic
WBC $x10^9/L$	0-0.2	0-5	2-200	50-200	0.5-200	0.05-10
Neutrophils (%)	<25	<30	>50	>90	<90	<50
Crystals present	No	No	No	No	Yes	No
RBCs present	No	No	No	Yes	No	Yes
Blood-fluid glucose ratio	0-10	0-10	0-40	20-100	0-80	0-20
Culture	Negative	Negative	Negative	Often Positive	Negative	Negative

13. SEMEN ANALYSIS

Semen consists of spermatozoa suspended in plasma like fluid and is formed at ejaculation. Only the spermatozoa and a small amount of secretions are produced in the testis and the epididymis (5% of total volume). Bulk of the semen consists of the secretions of seminal vesicles (46-80%) and prostate (13-33%). Bulbourethral and urethral glands contribute about 2-5% of total volume. These secretions not only affect the concentration but also the function of the spermatozoa. During intercourse each component is expelled in posterior urethra by the process of emission followed by ejaculation out of urethra. Mixing of the components takes place after ejaculation.



Various parts of testis contribute spermatozoa, androgens, androgen binding protein, transferrin and inhibin. Initially the sperms are not motile but while passing through the epididymis they acquire motility by virtue of carnitine that is added to the seminal fluid in epididymis. In addition, inositol, lipids and phospholipids are also added to semen at this site. Further, the ability to penetrate eggs is also acquired in the epididymis.



Seminal vesicles provide an important energy source in the form of fructose that enhances motility of spermatozoa. Prostaglandins and fibrinogen like substances are also added to seminal fluid by seminal vesicles. Prostatic fluid provides a number of enzymes, spermine (a bacteriostatic substance), citrates, calcium and zinc. The bulbourethral and urethral glands contribute mucoproteins and IgA to semen. Thus, disease of any of these parts of the male genital tract may have a profound effect on both the quality and the quantity of semen and may lead to infertility.

INDICATIONS FOR SEMEN ANALYSIS

These include Infertility, hypogonadism, follow up after vasectomy, prior to donations for artificial insemination and storage of semen before radiotherapy etc.

SAMPLE COLLECTION

A period of abstinence is important as it affects both the quantity and motility of spermatozoa. Ordinarily abstinence for 3-5 days is adequate. It is more convenient and practical to produce the specimen in the laboratory. However, some patients may not feel relaxed and comfortable in the laboratory atmosphere and stress is known to affect both the quality and the quantity of semen. In such cases it might be more fruitful to ask the patient to produce the specimen at home and quickly transport it to the laboratory.

The specimen should be collected in the morning to allow sufficient time for its analysis. Masturbation is the ideal method for producing the semen specimen. However, due to psychological or religious reasons this might not be possible in some patients. In such instances coitus interruptus can be resorted to but a part of the ejaculate may be lost by this method. It is important that both the pathologist and the patient be aware of this fact. Condoms must never be used for collection of semen by intercourse. A clean, dry, wide-mouth glass or plastic jar should be used as semen container. Its lid must not be rubber lined. Detergents, water and rubber are injurious to sperms. Specimen should be transported to the laboratory at a temperature as close to 37°C as possible and delivered to the laboratory in less than 2 hours.

PHYSICAL EXAMINATION

Transfer the semen into a scrupulously clean graduated small cylinder. Note the volume, colour, appearance and the pH. Normally, the human semen, soon after ejaculation, forms a gel-like clot that liquefies in 5-20 min and

therefore, by the time it is brought on the workbench it has usually liquefied. If not, it should be liquefied before analysis by adding 5-10 drops of 0.2% α-amylase. Absence of liquefaction in a semen sample must be noted. Viscosity of semen should be assessed. It can be measured by dropping a drop of semen from a 10 cm long capillary tube containing 0.1 ml semen. Time taken by the drop to form and leave the capillary tube is a measure of its viscosity.

SPERM COUNTING

Visual assessment

Place a drop of semen on a clean glass slide and lightly place a cover slip over it. Examine the slide under the high power objective of a microscope to make a visual assessment of the sperm count and to determine the need for any dilution.

Dilution

The diluent used is 3.5% buffered formal saline prepared by dissolving 5 g sodium bicarbonate, 1 ml of 35% formalin and distilled water to make a total volume up to 100 ml. Five ml of saturated aqueous solution of gentian violet can be added to this fluid to stain the sperms. The fluid immobilises the spermatozoa and facilitates counting. Normally 1 in 20 dilution is made by adding 50 µl of well-mixed and liquefied semen to 950 µl of diluent (Sahli pipette). However, 1 in 10 dilution is recommended and 1 in 50 dilution may be required if sperm count appears high.

Counting procedure

Improved Neubauer chamber (Haemocytometer) is used for counting. Both the chamber and the cover slip must be washed with distilled water and dried before use. The cover slip is then pressed on the central area until all the air is out and birefringent rings appear on the side. The diluted semen is carefully mixed and the chamber is charged using a Pasteur pipette. The chamber is then examined by using x10 objective of microscope. Sperms are counted in the four large corners and one large central square (WBC counting area) (page 253). It is important that loose tails and germinal cells are not counted. At least 200 spermatozoa must be counted. If these are not available in these 5 squares, more squares must be counted.

Calculation

$$\text{Sperm count (million/ml)} = \frac{CxDx1000}{V}$$

Where

C = Count in 5 large squares
D = Dilution factor
V = Vvolule of 5 large s1ures
1000 = To convert mm^3 into ml

ASSESSMENT OF SPERM MOTILITY

Assessment of motility must be performed soon after production of sample, 3 and 6 hours later. It is important to remember that sperms require at least 10 µm of depth for free movement. A drop of well-mixed undiluted semen is placed on a warm clean slide and very lightly covered with a cover slip. The slide is allowed to rest on the microscope stage or bench until 'streaming' of the semen stops and is then viewed under the microscope. Both motile and immotile sperms are counted at least in 5 fields with a minimum count of 200. The count should be performed in duplicate and the average recorded. Only forward movement of the sperms is taken as positive. Percentage motility is then calculated. The sperm count can be calculated using the formula:

$$\text{Motile sperm count} = \frac{\text{Sperm count/ml} \times \%\ \text{motility} \times \text{semen vol}}{100}$$

More objective results can be obtained by following procedure:

1. About 30 min after collection transfer the semen in a capped tube. Gently mix by inverting the tube several times.
2. Pipette one drop of semen onto a clean glass slide; place a clean cover slip over it.
3. Observe with a x40 objective and estimate the percentage of spermatozoa moving at following speeds:
   - Grade 0: No movement at all
   - Grade 1: Moving with no forward progression
   - Grade 2: Moving with slow and wandering movement
   - Grade 3: Moving rapidly in almost straight line
   - Grade 4: Moving with high speed in straight line

Calculate a motility score by adding up the product motility grade and percentage of spermatozoa in that grade. Example is as under:

Table 13.1: Calculation of motility score.

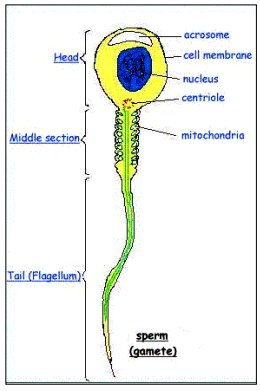
Grade	x	Percentage	=
0	x	30	0
1	x	10	10
2	x	15	30
3	x	30	90
4	x	15	60
Total score			190

Normal motility score for spermatozoa is ≥150. Motility depends upon temperature. At 37°C only

50% sperms are motile after 3 hours. At 21°C 50% are still motile after 7 hours. However, temperature below 20°C decreases the motility.
Artefactual asthenozoospermia can be produced by contamination of the container with water, soap, detergents, or after contact with rubber.
Asthenozoospermia caused by cold exposure (<20°C) of the semen sample, infection or fructose deficiency can be easily diagnosed by performing the following simple test:
Exposure to cold: Return of sperm motility after placing the semen sample for 30 min in the incubator is diagnostic of reduced motility due to cold.
Infection: Manifested by the presence of excess white cells or bacteria. Bacterial culture will help.
Fructose deficiency: Addition of an equal volume of warm Bakers buffer (3.0g glucose, 0.46g Na_2HPO_4 $7H_2O$, 0.2g NaCl, 0.01g KH_2PO_4 and distilled water up to 100 ml) to an aliquot of semen on a glass slide will produce a resumption of sperm motility if due to fructose deficiency.

ASSESSMENT OF SPERM MORPHOLOGY

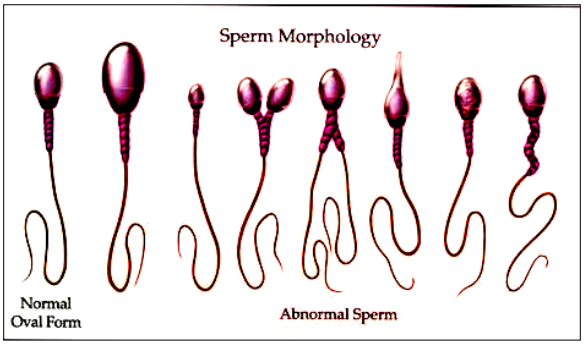
A normal sperm consists of a head and a tail joined together by a short neck. The head is oval in shape and measures 4.5x3x1.5 µm while the tail is about 50 µm long (10 times the head and neck length). The tail comprises a mid-piece, the principal piece and a terminal segment. Most of the tail length (90%) is composed of the principal piece. Assessment of morphology can be made in a wet preparation or in a stained smear of semen. As it is difficult to define morphology in a motile sperm, it is better to use stained smear. For staining, smear is made in the same way as blood smear is made. It can then, be stained by haematoxylin and eosin or Papanicolaou or May-Grunwald-Giemsa stains. The slides are then examined under oil immersion objective of the microscope. Abnormalities of the head including small, large, tapering, pyriform, amorphous and double heads; of the tail including double, coiled or short tails and of the mid-pieces should be noted. At least 100 spermatozoa must be examined, the percentage of abnormal sperms should be stated and morphological abnormalities described. Also make a note of the presence of white blood cells, epithelial cells, red blood cells, germinal cells, lymphocytes, extraneous particles, protozoa and bacteria.



REPORTING

Some of the special terms used for reporting the results of semen analysis are:

- Aspermia: No ejaculate.
- Oligospermia/Hypospermia: Reduction in volume of ejaculate.
- Hyperspermia: Increase in volume of ejaculate.
- Oligozoospermia: Low sperm count (<30 million/ml).
- Polyzoospermia: High sperm count (>300 million/ml)
- Asthenozoospermia: Absence or marked reduction in sperm motility (Motility score <150)
- Oligoasthenozoospermia: Low count with low motility.
- Necrospermia: Dead sperm



REFERENCE RANGE

Volume	2-6 ml (1-10 ml)
Colour	Grey-yellow
Appearance	Opalescent
Viscosity	Viscous
pH	7.2-8.9
Sperm count	60-150 million/ml (Extreme range 30-300 million/ml)
Motility	>70% at 1 hour and 50% at 3 hours after ejaculation
Motility score	≥150
Morphology	>70% should be morphologically normal

TESTING FOR ANTI-SPERM ANTIBODIES

Testing for anti-sperm antibodies is as important in the evaluation of infertile male as the semen analysis and individual laboratories can without much difficulty incorporate these tests in their routine work. Agglutination tests, sperm immobilising antibody tests, testing for cytotoxic antibodies are the various methods for demonstrating sperm antibodies.

Procedure

Separation and preparation of donor sperms

1. Layer 2 ml RPMI[1] 1640 with 5% FCS[2] over semen.
2. Incubate at 37°C for 30 minutes.
3. Take off the upper most 2 ml.
4. Examine under the microscope for motility.
5. Wash once with RPMI 1640 with 5% FCS.
6. Adjust count to 2000/µl.

Testing antisperm agglutinating and immobilising antibodies

1. Inactivate complement in test and normal serum by incubating at 56°C for 30 minutes. Proceed according to Table 13.2.
2. Pipette 1 µl of 5% FCS made in RPMI 1640 in all the wells of control and test except column A of microtitration plate.
3. Dispense 1 µl normal serum in column A of rows 1 and 2.
4. Dispense 1 µl test serum in column A of rows 3 and 4 so two rows are used for each test serum.
5. Prepare doubling dilutions of the test and normal serum in each row with 5% FCS made in RPMI 1640 i.e., 1:2, 1:4, 1:8, 1:16, 1:32.
6. Add 1 µl donor sperms in each well.
7. Mix well on shaker for 2 minutes.
8. Incubate at 37°C for 30 minutes.
9. Observe under microscope for agglutination.

Table 13.2: Worksheet for testing antisperm antibodies

	A	B	C	D	E	F		
1								Normal control serum
2	//	X	X	X	X	X		
3								Test 1
4	//	X	X	X	X	X		
5								Test 2
6	//	X	X	X	X	X		
7								Test 3
8	//	X	X	X	X	X		
9								Test 4
10	//	X	X	X	X	X		
	1/1	1/2	1/4	1/8	1/16	1/32		

10. Add 2 µl Rabbit complement in rows 2,4,6,8,10 (the crossed rows) in all wells, leaving the well A to see the antisperm immobilising antibodies.
11. Incubate 37°C for 1 hour.
12. Observe under microscope for agglutination.

FRUCTOSE TEST

Fructose is absent from the semen of patients with bilateral aplasia of the vasa differentia and seminal vesicles. It is also absent in bilateral obstruction of the ejaculatory ducts.

Reagents

Resorcinol reagent is prepared by adding 33 ml HCl to 50 mg resorcinol and then making up the volume to 100 ml by addition of distilled water.

Procedure:

Place 0.1 ml of semen in a test tube. Add to it 1 ml of resorcinol reagent. Boil for 5-10 min. The solution turns reddish brown in the presence of fructose. No change in colour indicates absence of fructose from the semen.

IMPORTANT NOTES

- An important cause of aspermia is retrograde ejaculation (ejaculation backwards into urinary bladder). In all cases when ejaculate is not obtained, a urine specimen should be immediately collected and examined for spermatozoa.
- An immotile sperm does not necessarily mean a dead sperm. It is important to distinguish between asthenozoospermia and necrospermia. For this mix a drop of semen with a drop of 0.5% yellow eosin in distilled water on a glass slide. Place a cover slip and examine under microscope. Dead spermatozoa will take a pink yellow colour while immotile living sperms remain unstained.
- For evaluating infertility, semen analysis should be performed on three occasions with a gap of 2-3 weeks between any two analyses.

[1] RPMI = Rose Parkwell Memorial Institute

[2] FCS = Foetal calf serum

SECTION III – PARASITOLOGY

14. PARASITOLOGY

CLASSIFICATION

Parasitology is the science dealing with parasites and their pathogenicity. A **parasite** is a living organism that has adopted itself to exist in another animal called **host**. Parasitic infestations in man constitute the most common health problem, particularly in tropical and developing countries. Parasites infest man in more than one tissue and organ. The methods employed to investigate such infestations depend upon the biological behaviour of the parasite, the organ that it involves and its method of reproduction and transmission. Numerous parasites are capable of infecting man depending upon:

1. Its presence in a geographical area (endemicity).
2. Suitable climate for propagation.
3. Presence of intermediary hosts (if required).
4. Presence of vector (if required) for its transmission.
5. Habits of the people.
6. Hygienic status of the society.

Table 14.1: Parasites, Classification and Sites of Infection.

Class	Species	Site
Protozoa		
Amoebae	*Entamoeba histolytica*	Large, intestine, liver lungs etc.
	Naegleria fowleri	Brain and CSF
	Acanthamoeba spp	Brain and CSF
Flagellata	*Giardia Lamblia*	Duodenum and gall bladder
	Trichomonas hominis	Colon
	Trichomonas vaginalis	Vagina
	Leishmania tropica	Skin
	Leishmania braziliensis	Skin and mucous membrane
	Leishmania donovani	Reticuloendothelial system particularly liver, spleen and bone marrow.
	Trypanosoma brucei-gambiense	All tissues, blood, CNS
	Trypanosoma brucei-rhodesiense	All tissues, blood, CNS
	Trypanosoma cruzi	Myocardium and smooth muscle of the gut.
Ciliata	*Balantidium coli*	Intestine (non pathogenic)
Sporozoa	*Plasmodium vivax*	Blood and liver
	Plasmodium ovale	Blood and liver
	Plasmodium malariae	Blood and liver
	Plasmodium falciparum	Blood
	Toxoplasma gondii	All tissues particularly lungs and brain.
	Isospora belli	Intestine (opportunistic pathogen)
	Babesia microti	Skin and blood stream
	Cryptosporidium parvum	Mouth, small intestine and other mucosal surfaces
	Microsporidium spp	Mouth, small intestine and eye
	Pneumocystis jirovi (formerly *P,carinii*	Lungs, bone, eye, lymph nodes, adrenal glands, GIT, kidney, thyroid
Helminths		
Nematoda (Round worms)	*Ascaris lumbricoides*	Small intestine and lungs
	Toxocara canis	All organs and tissues
	Toxocara cati	All organs and tissues
	Anisakis spp	Gastric, duodenal and jejunal mucosa.
	Ancylostoma duodenale	Duodenum and jejunum
	Necator americanus	Duodenum and jejunum
	Strongyloides stercoralis	Duodenum, jejunum, lungs
	Enterobius vermicularis	Terminal ileum, colon
	Trichinella spiralis	Small intestine
	Wuchereria bancrofti	Lymphatics
	Onchocerca volvulus	Skin, eye, hip joint
	Loa loa	Skin, eye
	Dracanculus medinensis	Skin, Sub-peritoneal cavity
Cestoda (Tape worms)	*Taenia saginata*	Small intestine
	Taenia solium	Small intestine
	Hymenolepis nana	Ileum
	Diphyllobothrium latum	Small intestine
	Echinococcus granulosus	Liver, lungs and other tissues
	Echinococcus multilocularis	Liver, lungs and other tissues
Trematoda (Flukes)	*Fasciolopsis buski*	Small intestine
	Fasciola hepatica	Mouth, liver and biliary tract
	Schistosoma haematobium	Venous plexus of urinary tract
	Schistosoma mansoni	Haemorrhoidal plexus, liver, spleen
	Schistosoma japonicum	Superior mesenteric vein tributaries
	Colonorchis sinensis	Bile duct
	Paragonimus westermani	Intestine, lungs.

In this chapter important parasites will be enumerated. Only those will be discussed which are prevalent in Pakistan or carry some importance for our people working in other countries. Parasites can be classified according to the organs which they involve such as intestinal parasites, haemoparasites etc., or according to their taxonomy. In this chapter attempt has been made to mix these classifications for the purpose of better

understanding.

PROTOZOA

Protozoa can be defined as unicellular organisms that are independently complete. They can eat, respire, move and reproduce without help. They are divided into four classes as shown in Table 14.1.

HELMINTHS

Helminths are multicellular organisms of varying sizes, elongated in shape and having a reproductive system. Other system like nervous system and gut may be present in a rudimentary form. Only a few parasites occur in Pakistan and even fewer are important pathogens. They may infect man in their adult or larval forms. These diseases, although, may prove fatal in certain cases, but are easy to treat and are curable provided these can be diagnosed. In the next few pages, life cycles and methods of diagnosis of some important parasites will be discussed.

MALARIA

Malaria is one of the most widely spread parasitic disease of the world. It mainly occurs in tropical and subtropical areas but cases are found all over the world due to travelling to and from these areas. A protozoan belonging to the class sporozoa and the genus plasmodium causes it. Four species are involved namely, *P.vivax*, *P.ovale, P.malariae* and *P.falciparum*. All species differ in morphology, life cycle and type of disease they cause. The parasite invades and destroys red blood cells. It is transmitted from one person to another through bites of a mosquito of the genus anopheles.

LIFE CYCLE

Life cycle of malarial parasite involves two hosts and consists of a **sexual cycle or sporogony** in mosquito and an **asexual cycle or schizogony** in man. Man is actually the intermediate host while mosquito is the definitive host (Figure 14.1).

ASEXUAL CYCLE IN MAN (SCHIZOGONY)

During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host 1. Sporozoites infect liver cells 2 and mature into schizonts 3, which rupture and release merozoites 4. This is **pre-erythrocytic schizogony** or tissue phase. (Of note, in *P. vivax* and *P. ovale* is a dormant stage [hypnozoites] that can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later also called as **exo-erythrocytic stage**). After this initial replication in the liver A, the parasites undergo asexual multiplication in the erythrocytes (**erythrocytic schizogony** B). Merozoites infect red blood cells 5. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites. 6 When the infection is well established, some merozoites differentiate into sexual erythrocytic stages (**gametocytes**) 7 after about 12 days. Blood stage parasites are responsible for the clinical manifestations of the disease. The length of erythrocytic cycle and the number of asexual generations varies depending upon the species. If large numbers of red cells rupture simultaneously, a malarial paroxysm results from the toxic material released into the bloodstream. The time taken to complete this cycle varies in different species. In *P.vivax* it is 45 hours, in *P.ovale* 48 hours, in *P.malariae* 72 hours and in *P.falciparum* 48 hours. Fever occurs at the time of liberation of merozoites.

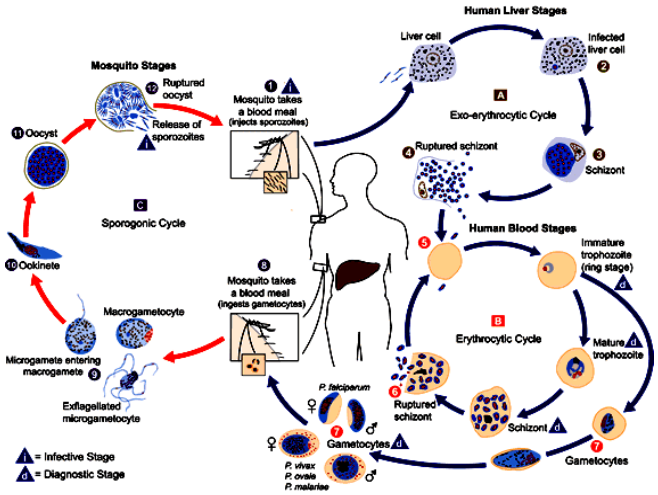


Figure 14.1: Sexual and asexual life cycle of Plasmodium species

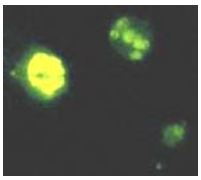
SEXUAL CYCLE

The sexual forms of the parasite the gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal 8. The parasites' multiplication in the mosquito is known as the **sporogony** C. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes 9. The zygotes in turn become motile and elongated (ookinetes) 10 which invade the midgut wall of the mosquito where they develop into oocysts 11. The oocysts grow, rupture, and release sporozoites 12, which make their way to the mosquito's salivary glands. Inoculation of the

sporozoites into a new human host perpetuates the malaria life cycle [1]. All sexual and asexual forms of the parasite described in human cycle are seen in peripheral blood except in *P.falciparum* where most of maturation occurs in RBCs sequestered in small vessels. In this case only ring forms and gametocytes are seen in the blood. It is important to identify and report *P.falciparum* because it not only gives rise to immediate serious complications but may also be resistant to ordinary drugs. The incubation period varies from 8-11 days in *P.falciparum* to 18-40 days in *P. malariae*. However sometimes it may be prolonged for months to years.

LABORATORY DIAGNOSIS

The diagnosis depends upon demonstration of the parasite in blood (Figure 14.2). **Thick smear** is used as a screening test, whereas the **thin smear** is to identify the species). Two stains are used. Leishman stain is prepared in alcohol which also acts as fixative, so both fixation and staining occur at the same time. On the other hand, in Giemsa staining the fixative and stain are separate; thus the thin film must be fixed prior to staining. Fluorescent dye may also be used. But this employs the use of fluorescence microscope. The immunodiagnostic procedures include indirect fluorescent antibody technique, indirect haemagglutination and parasite DNA detection by PCR but are not used in routine. The best time for collection of a blood sample is 6-12 hours after the onset of a chill as the blood at this time will contain larger number of trophozoites. It should be repeated 8 hours later to see mature trophozoites that are species specific. It is best to use fresh non-coagulated capillary blood, obtained by a prick. EDTA is preferred but heparin can also be used. Thick and thin films can be made on the same slide as shown in Figure 14.2.



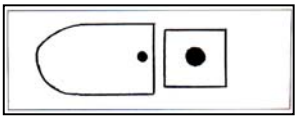


Figure 14.2: Size of blood drop and area of slide to cover for making thick and thin blood films

THICK FILM

Principle
A large amount of blood can be examined for parasitic forms by lysing the red cells and staining for parasite. Fixation is not done by methanol.

Procedure
Touch a large drop of blood from pulp of finger with a glass slide and rotate it to spread blood in an area equal to a two-rupee coin. Film should be such that newsprint can be seen through it. Alternatively, place a drop of blood in the centre of glass slide and spread it with a corner of another glass slide. Dry the blood film for 30 min at 37°C or leave it on top of a microscope lamp for about 7 min. Dilute stock Giemsa stain 20 times in buffered water in a staining jar and immerse the slide in it for 20-30 min. Take out and gently wash with buffered water and let stand upright to dry. The slide must not be blotted. Examine under oil immersion lens.

THIN FILM

Principle
By spreading the blood cells in a thin layer, the size of red cells, inclusions, and extracellular forms can be more easily visualised. Leishman stain is prepared in methanol which acts as fixative also.

Procedure
Slides are prepared in the usual manner and stained in the same way as for differential leukocyte count and red blood cell morphology (for details see PREPARATION AND STAINING OF BLOOD FILMS on page 256). More time should be spent on examination of the edges and head end of the slide.

MALARIAL PARASITE INDEX

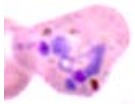
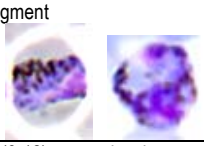
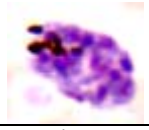
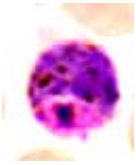
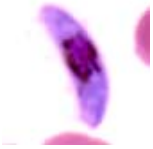
It is the degree of parasitaemia and is important to define response to treatment and resistance to anti-malarial drugs in malarial infection, particularly with *Plasmodium falciparum*. It can be calculated by following methods:

Thin film Procedure
Select an area with uniform distribution of RBCs. Count 500 RBCs noting the number of RBCs containing parasite. Calculate the index by dividing the number of parasitised RBCs with 5.

Thick film Procedure
Determine total WBC count. (page 253). Count systematically 100 WBCs, simultaneously counting the number of parasites in the same area. Repeat the counting procedure on two more areas of the same film. Calculate the average number of parasites per 100 WBCs. Index can be calculated by:

$$\text{WBC count}/\mu\ell \times \text{Parasite count}/\mu\ell \,/\, 100$$

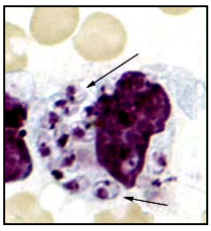
Table 14.2: Species Characteristics of Malarial Parasites

Form	*P. vivax*	*P. ovale*	*P.falciparum*	*P. malariae*
Ring form	1/3 of cell diameter, single, heavy, chromatin dot 	Similar to vivax but larger and more amoeboid 	Delicate, small, 1-2 dots, more than one in a cell, at the edge of Cell (applique) or drawn into a filament (accole form) 	Ring often smaller than *P.vivax* occupying 1/6 of cell heavy chromatin dot; pigment forms early. 
Trophozoites	Amoeboid, small vacuoles, fill the cell, fine brown pigment, stream of cytoplasm close to large chromatin dot 	Ring usually maintained until late 	Usually not seen 	Non-amoeboid, rounded or band shaped, solid forms; chromatin may be hidden by the coarse dark brown pigment 
Mature Schizonts	16 (12-24) merozoites, fill, entire RBC. Each has cytoplasm and chromatin dot 	¾ of cell occupied by 8 (8-12) merozoites, in rosette or irregular clusters, brown pigment in centre 	Rarely seen in peripheral blood 	8 (6-12) merozoites in rosettes or irregular clusters filling normal sized cells, central green-brown pigment 
Macrogametocytes	Rounded or oval homogenous cytoplasm, with diffuse delicate light brown pigment. Large pink chromatin mass surrounded by colourless halo, evenly distributed pigment 	Similar to *P.vivax* 	Sex differentiation difficult; crescent or sausage shaped; may appear in showers; black pigment near chromatin dot, which is often central 	Similar to *P.vivax* but less in number, pigment darker and coarser 
Microgametocytes	Large pink to purple chromatin mass surrounded by pale or colourless halo; evenly distributed 	Similar but smaller than *P.vivax* 	Like macrogametocytes 	Similar to *P.vivax* but less in number, pigment darker and coarser 
Main differential Criteria	Large pale red cell; trophozoites irregular; pigment usually present; Schuffner's dots not always present; several phases of growth seen in one smear; gametocytes appear early.	Red cell enlarged, oval with fimbriated edges; Schuffner's dots seen all stages.	Develop in blood vessels in internal organs; delicate ring forms and crescent shaped gametocytes seen in peripheral blood.	Red cell normal in size and colour; trophozoites compact, stain usually intense, band form not always seen; coarse pigment; no stippling of red cells; gametocytes appear late

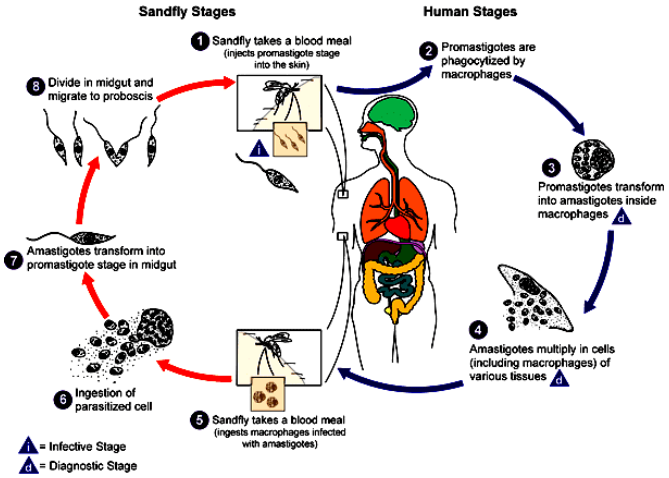
LEISHMANIASIS

CUTANEOUS LEISHMANIASIS

Cutaneous leishmaniasis is prevalent in eastern Baluchistan and southern Punjab. Cases have also been reported from NWFP and Kashmir. A flagellate protozoan *Leishmania tropica* complex causes the disease. The parasite is transmitted from man to man by sand fly of genus phlebotomus, which is the definitive host. Man is the intermediate host. The parasite exists in 2 different morphological forms in its life cycle. In man it occurs in the **Leishmanial (amsatigote)** form. It is ovoid in shape, measuring 1.5-5 µm. It contains a nucleus and close



to it much smaller structure called the kinetoplast. In the body of the sandfly it is transformed into **leptomonad** (**promestigote**) form that is large elongated and has a polar flagellum in addition to a nucleus and kinetoplast. Leishmaniasis is transmitted by the

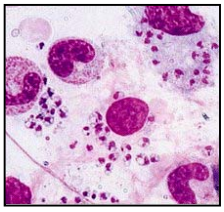


bite of female phlebotomus. The sand flies inject the infective stage, promastigotes, during blood meals(1). Promastigotes that reach the puncture wound are phagocytosed by macrophages (2) and transform into amastigotes(3). Amastigotes multiply in infected cells and affect different tissues, depending in part on the *Leishmania* species(4). This originates the clinical manifestations of leishmaniasis. Sandflies become infected during blood meals on an infected host when they ingest macrophages infected with amastigotes ((5),(6)). In the sandfly's midgut, the parasites differentiate into promastigotes(7), which multiply and migrate to the proboscis(8).

LABORATORY DIAGNOSIS

The diagnosis made by examination of a smear from the lesions, culture of material from the lesion, and biopsy. The easiest way is to examine a Giemsa or Leishman stained smear prepared from material obtained from the lesion. Smear can be prepared by any method given below:

- Clean the edge of the ulcer and surrounding skin. Give a small, skin-deep incision with a sharp blade, about 5 mm in length starting from ulcer margin. Spread the material onto a clean glass slide.
- Take a corrugated dental needle and insert it into the skin at the margin of the ulcer pointing towards the floor of the ulcer. Withdraw the needle without rotating. Spread the material sticking to the needle on a clean glass slide.
- Take a 50 ml syringe and attach to it a long wide bore needle. Clean the skin around the ulcer. Introduce the needle into the skin about 1 cm away from the ulcer. Penetrate subcutaneous tissue in the direction of the ulcer. When the tip reaches below the ulcer margin apply suction until an exudate appears in the hub of the needle opening inside the syringe. Remove the syringe from the needle while maintaining suction. Withdraw the needle. Fill syringe with air and reattach to needle. Blow out contents of needle onto clean glass slides and prepare smears.



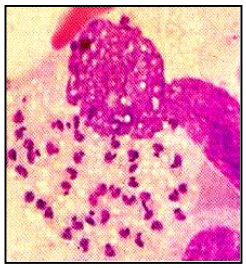
Stain smears just like blood smears and examine under high power objective (x40). Look for large macrophages with parasites and study morphology of parasites under oil immersion lens. It is important to demonstrate:

- Intracellular parasites, and
- Both nucleus and kinetoplast inside the parasite

It is difficult to obtain satisfactory specimens in lesions secondarily infected with pyogenic bacteria. It is difficult to identify parasites in such smears due to presence of bacteria and other inclusions. It is better to repeat the smear for parasite after treating the bacterial infection. The parasites are also called LT (*Leishmania tropica*) bodies. The specimens can be cultured on artificial media (page 114). Montenegro skin test is positive in high percentage of cases. Indirect fluorescent and ELISA techniques have been developed for diagnosis of cutaneous leishmaniasis.

VISCERAL LEISHMANIASIS

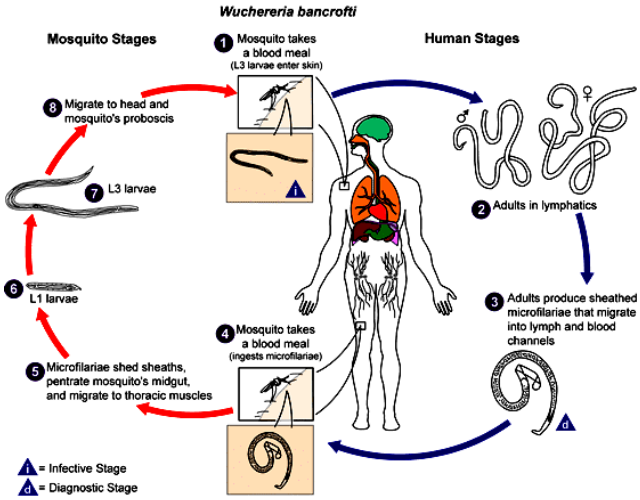
Commonly called Kala Azar, it is seen in Pakistan, particularly in Azad Kashmir and Baltistan areas. It is caused by at least three subspecies belonging to the *Leishmania donovani* complex, clinically and biochemically distinct having different geographic distribution. *Leishmania donovani* is transmitted through the bites of sand fly (phlebotomus). The life cycle is similar to *Leishmania tropica* except that, in this case the parasite attacks the reticuloendothelial system of liver, spleen and bone marrow. The disease is commonly diagnosed by demonstration of



intracellular parasites in material obtained by splenic puncture or in bone marrow aspirates. The parasite may also be seen in liver biopsy specimen occasionally showing macrophages containing LD bodies (*Leishmania donovani* is similar to *Leishmania tropica).* Buffy coat films prepared from venous blood are sometimes of value. Culture of *Leishmania* is possible on Schneider's Drosophilia, RPMI medium 1640 and NNN medium. For animal pathogenicity intraperitoneal inoculation in hamsters is used. Montenegro (leishmanin) skin test, antibody detection by ELISA and immunofluorescence techniques is also available for diagnosis.

FILARIASIS

Microfilariae are the larvae of nematodes. The filarial worms are long and thin that inhabit lymphatic system and subcutaneous and deep connective tissues. Most species produce microfilariae, which can be found in the peripheral blood; two species, *Onchocerca volvulus* and *Mansonella streptocerca*, produce microfilariae found in subcutaneous tissues and dermis. Microfilariae can cause serious diseases



like elephantiasis and blindness. Only filariasis (Elephantiasis) caused by *Wuchereria bancrofti* occurs in Pakistan. Other species are rare. Man is the definitive host while the mosquito of genus *Culex* is the intermediate host. Sexes are separate. The parasite occurs in couple pairs and obstructs lymphatics resulting in elephantiasis. During a blood meal, an infected mosquito introduces third-stage filarial larvae onto the skin of the human host, where they penetrate into the bite wound[1]. They develop in adults that commonly reside in the lymphatics[2]. The female worms measure 80-100 mm in length and 0.2-0.3 mm in diameter, while the males are half the size of females. Adults produce microfilariae measuring 240-300 µm by 7.5-10 µm, which are sheathed and have nocturnal periodicity. The microfilariae migrate into lymph and blood channels moving actively through lymph and blood[3]. A mosquito ingests the microfilariae during a blood meal[4]. After ingestion, the microfilariae lose their sheaths and some of them work their way through the wall of the proventriculus and cardiac portion of the mosquito's midgut and reach the thoracic muscles[5]. There the microfilariae develop into first-stage larvae [6] and subsequently into third-stage infective larvae[7]. The third-stage infective larvae migrate through the haemocoel to the mosquito's proboscis [8] and can infect another human when the mosquito takes a blood meal[1].

LABORATORY DIAGNOSIS

Diagnosis of filarial infections is often based on clinical ground, but demonstration of the parasite is the only accurate means of confirming the diagnosis. Blood should be collected around midnight, as this is the time when parasite is present in the blood (Figure 14.3). There are three methods of examination:

- Prepare an ordinary thin blood smear and stain in the usual manner. Examine under low power and then for finer details, under high power.

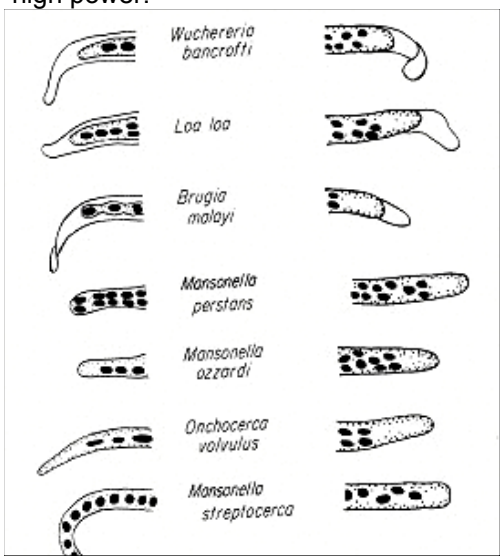


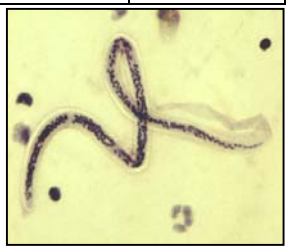
Figure 14.3: Cephalic and tail ends of various filariae

- Make a thick blood film stained with Giemsa stain. Better results are obtained with haematoxylin and eosin staining. For this the dried smear is first washed with water, dried in air and fixed with equal parts of ether and 95% alcohol for 10 min. It is dried and stained like histological sections.

- In concentration method, capillary blood is obtained in a centrifuge tube containing 2% acetic acid. It is mixed thoroughly, centrifuged and the deposit examined under a cover slip. Actively moving microfilariae can be observed.

Periodicity of microfilariae in circulation	Species
Nocturnal	*W.bancrofti* *B.malayi* *B.timori*
Diurnal	*Loa loa*
Aperiodic	*Mansonella*

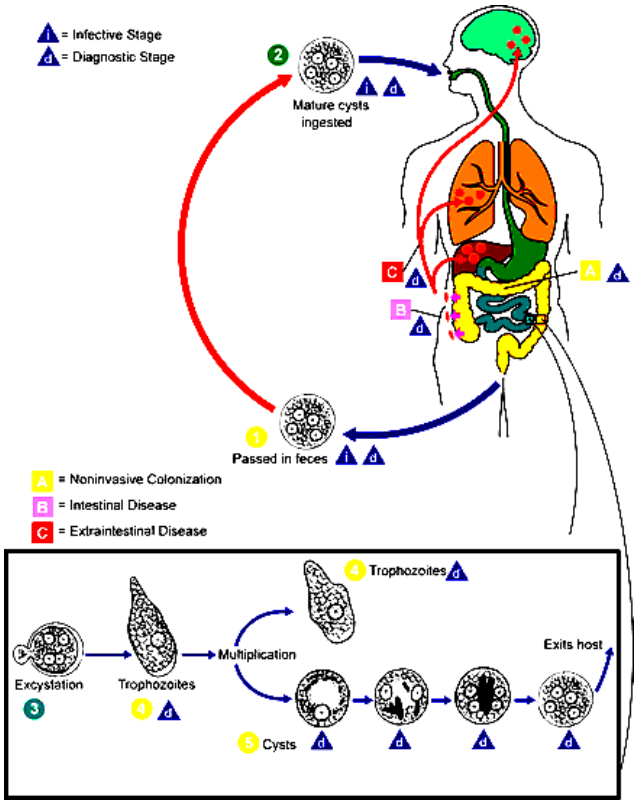
The microfilariae of *O.volvulus* and *D.streptocerca* are found in **skin snips**, very thin slices of skin, which are teased apart in normal saline to release the organisms.



INTESTINAL PARASITES

AMOEBIASIS

This disease is caused by the protozoan *Entamoeba histolytica*. Out of seven amoebae occurring in nature, it is the most common pathogen of man, second to malaria as cause of death due to parasitic protozoa. It occurs world-wide but infests about 10% of population. Prevalence in tropical countries may be more than 30%. Cysts are passed in faeces 1. Infection by *Entamoeba histolytica* occurs by ingestion of mature cysts 2 in faecally contaminated food, water, or hands. Excystation 3 occurs in the small intestine and trophozoites 4 are released, which migrate to the large intestine. The trophozoites multiply by binary fission and produce cysts 5, which are passed in the faeces 1. Because of the protection conferred by their walls, the cysts can survive days to weeks in the external environment and are responsible for transmission. (Trophozoites can also be passed in diarrhoeal stools, but are rapidly destroyed once outside the body, and if ingested would not survive exposure to the gastric environment.) In many cases, the trophozoites remain confined to the intestinal lumen (A: **noninvasive infection**) of individuals who are asymptomatic carriers, passing cysts in their stool. In some patients the trophozoites invade the intestinal mucosa (B: **intestinal disease**), or, through the bloodstream, extraintestinal sites such as the liver, brain, and lungs (C: **extraintestinal disease**), with resultant pathologic manifestations. Transmission can also occur through faecal exposure during sexual contact (in which case not only cysts, but also trophozoites could prove infective). Two developmental stages are:

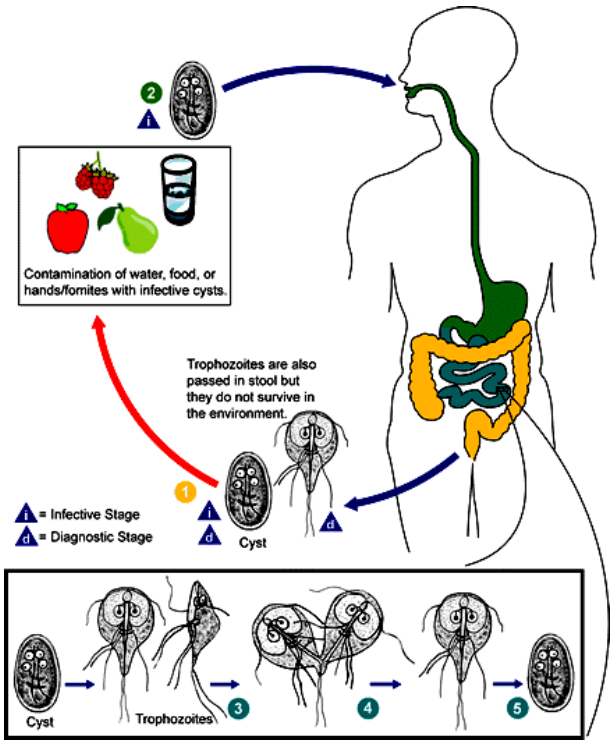


1. **The trophozoite stage** or vegetative form is the invasive form. It invades the intestinal wall causing a typical flask shaped ulcer in caecum and ascending colon, but other parts of the large intestine may also be affected. From intestine these may reach the liver via the portal circulation. The trophozoites are 20-60 µm in diameter. They are motile by explosive movements of pseudopodia. They ingest red blood cells, which is diagnostic. They have one nucleus and reproduce by binary fission.
2. **Cystic stage**: When the conditions are unfavourable the trophozoites become immobile, rounded and finally encyst. They may also divide within the cyst. Amoebic cysts thus may contain multiple nuclei. Cysts contain rod like structures called chromatoid bodies or bars and an inconspicuous glycogen vacuole. The cysts are passed in stool and may be ingested by another individual through contaminated food and water. Only four-cell stage cyst is infective. On reaching the intestine, the four nuclei divide to form 8 nuclei. Then the cyst wall disappears and 8 trophozoites are liberated which then attack the intestinal mucosa.

LABORATORY DIAGNOSIS

In acute amoebic dysentery the diagnosis is made by demonstration of trophozoites containing red blood cells showing typical unidirectional, purposeful movement. This can only be achieved by examining typical exudate from freshly passed faeces immediately. With exposure to cold, the amoebae become immobile and are difficult to distinguish. Trophozoites or vegetative forms of amoebae can be demonstrated in pus aspirated from liver or other abscess if examined immediately. The pus has characteristic **anchovy sauce** appearance. Amoebae may often be found in specimens obtained by sigmoidoscopy. Asymptomatic carriers and chronic cases often pass amoebic cysts. These can be identified in iodine stained preparation by the number of nuclei and shape of chromatid bars (page 91). *E.histolytica* may be cultivated in TYI-S-33 medium.

Serological identification: Indirect haemagglutination, indirect immunofluorescence, ELISA, complement fixation and gel diffusion tests are available. Direct immunofluorescence can demonstrate the amoebic antigens in stool specimens. DNA hybridisation probe also has been used to identify *E.histolytica* in stool specimens. However, false negative and positive results are common in serological tests.



GIARDIASIS

This disease is caused by a flagellate protozoan, *Giardia lamblia*. Infestation occurs in the upper small intestine and causes anaemia, weight loss and malabsorption. Diarrhoea and other abdominal symptoms may or may not occur. Parasite usually attaches to the intestinal mucosa and damages the brush border. Attachment of Giardia to the duodenal mucosa is facilitated by a lectin produced by the parasite and activated by duodenal secretions. Oedema and immunocyte infiltration of mucosa further increases the damage causing malabsorption. They may penetrate down into the secretory tubules of the mucosa and found at times in the gallbladder and in biliary drainage. The parasite is found in two forms. The **trophozoite** form is found in the intestine close to or on the microvillous border of the epithelium. Towards lumen and down in the intestine the conditions become unfavourable for trophozoites which then encyst. **Cysts** are excreted in stools. Occasionally, trophozoite forms may be seen in faeces if there is diarrhoea. Both cysts and trophozoites can be found in the faeces (**diagnostic stages**)1. Infection occurs by the ingestion of cysts in contaminated water, food, or by the faecal-oral route (hands or fomites)2. In the small intestine, excystation releases trophozoites (each cyst produces two trophozoites) 3 which remain in proximal small bowel4. Encystation occurs as the parasites transit toward the colon. The cyst is the stage found most commonly in non-diarrhoeal faeces5. Because the cysts are infectious when passed in the stool or shortly afterward, person-to-person transmission is possible. The parasite is found throughout the world. Children are more susceptible with a peak incidence around 10 years of age.

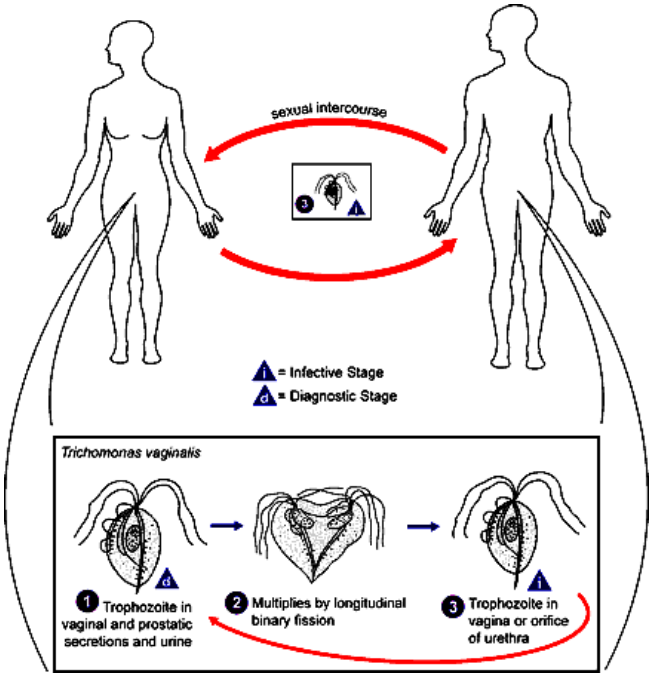
LABORATORY DIAGNOSIS

Diagnosis is made by demonstration of cysts in stools. A series of even 5-6 stools may be examined without recovering the organism, because it tends to pass on a cyclical basis and is securely attached to mucosa. Occasionally a typical trophozoite showing spinning movements may be seen in diarrhoeal stools. If suspicion is strong and cysts are not found in stools even on repeated examination, jejunal sampling is done. This includes jejunal aspirate or jejunal biopsy. Biopsy imprint or mucus attached to it is examined for the presence of trophozoites. Giardiasis may be diagnosed by detecting *Giardia* cysts or trophozoites in faecal

specimens (page 91), by ELISA or immunofluorescence and by detecting *Giardia* faecal antigen by counterimmunoelectrophoresis and ELISA. The entero test capsule can be helpful in recovering the organism as can the duodenal aspirate.

TRICHOMONAS VAGINOSIS

This protozoan is not an intestinal parasite but may contaminate faeces. Normal body sites include vagina and prostate. It is pathogenic for genital system and sometimes urinary tract. It is included in the list of sexually transmitted diseases (STD). Living trophozoite is 5-15 µm in



size but it may reach a length of 30 µm. They have very jerky and non-directional movement. It has four anterior flagella plus a recurrent flagellum that arises anteriorly and parallels the body, running to the posterior end. It forms the outer edge of the undulating membrane, a thin sheet of protoplasm that joins the body along a line marked by the presence of a curved, thin rod called the costa. The undulating membrane extends about half the distance to the posterior end of the body with no free flagellum. *Trichomonas vaginalis* resides in the female lower genital tract and the male urethra and prostate[1], where it replicates by binary fission[2]. The parasite does not appear to have a cyst form, and does not survive well in the external environment. *Trichomonas vaginalis* is transmitted among humans, its only known host, primarily by sexual intercourse[3].

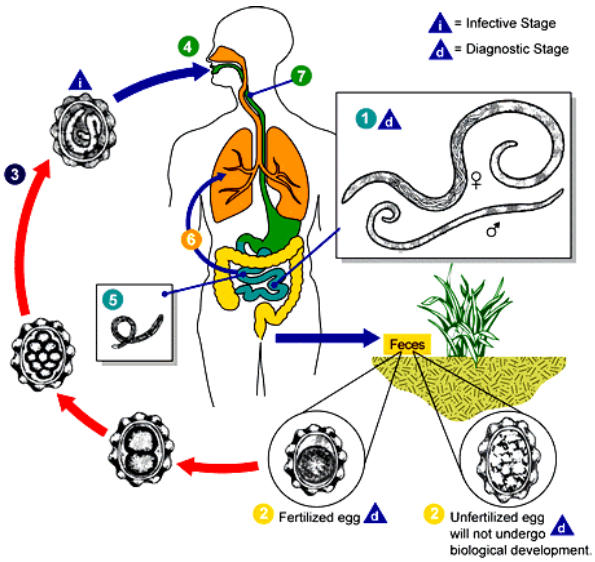
LABORATORY DIAGNOSIS

Diagnosis is by demonstration of trichomonas, most commonly in wet film preparation although they may readily be recognised in Papanicolaou smears. The most common specimen is vaginal discharge but examination of urethral discharge in the female may yield positive results when no organism is found in the vaginal swab. Culture can be made on modified Diamond's medium. Indirect haemagglutination test and Gel diffusion have been used for diagnosis of *T.vaginalis* infection, particularly for epidemiology. Monoclonal fluorescent antibody staining of clinical specimens has also been used for diagnosis. Culture techniques are better with sensitivity of 89% in Trichomonas medium No.2 and 97% with PEM-TV. Latex agglutination test is also satisfactory. Several specimens may needs to be examined. It is absolutely necessary that the specimen is NOT contaminated with faecal material since the morphology of *T. hominis* is similar to this organism.

ASCARIASIS

Ascariasis is caused by a large roundworm, *Ascaris lumbricoides* belonging to nematode. It is the most common intestinal helminth in man. Adult worms [1] live in the lumen of the small intestine. A female may produce approximately 200,000 eggs per day, which are passed in the faeces[2]. Unfertilised eggs are not infective. Fertile eggs embryonate and become infective after 18 days to several weeks[3], depending on the environmental conditions (optimum: moist, warm, shaded soil). After infective eggs are swallowed [4], the larvae hatch [5], invade the intestinal mucosa, and are carried via the portal, then systemic circulation to the lungs [6]. The larvae mature further in the lungs (10-14 days), penetrate the alveolar walls, ascend the bronchial tree to the throat, and are swallowed. [7] Upon reaching the small intestine, they develop into adult worms[1]. Between 2 and 3 months are required from ingestion of the infective eggs to egg production by the adult female. Adult worms live for 1-2 years. Infection commonly occurs in children playing on contaminated ground. Pica also causes infestation in the subtropics. Male and female worms are different and presence of both is necessary for passage of fertilised infective ova. Lung symptoms (pneumonitis, bronchial syndrome) are caused when the larvae are migrating from gut to respiratory system. Loss of appetite, nausea, vomiting and vague abdominal pain may occur. It may cause impaired intestinal

absorption and lactose insufficiency. More important acute complication occurs when either a bunch of parasite blocks the intestine or a parasite enters narrow passages like appendix, bile duct, or upper respiratory tract causing obstruction.



LABORATORY DIAGNOSIS

Worms: It is direct examination of a worm passed through anus or mouth**.** The adult worm is white or pink with fine striations on the cuticle. Posterior end of the male is curved. The male is 15-25 cm long and the female is 20-35 cm long. Both have 3-6 mm diameter.

Demonstration of ova in stools: Eggs are not passed if only a male worm is present in the intestine. If only a female worm is present then unfertilised ova are passed. For morphology see chapter on faeces examination (page 92).

Demonstration of larvae in sputum: These are 0.2-2.0 mm long, cylindrical in shape with pointed ends.

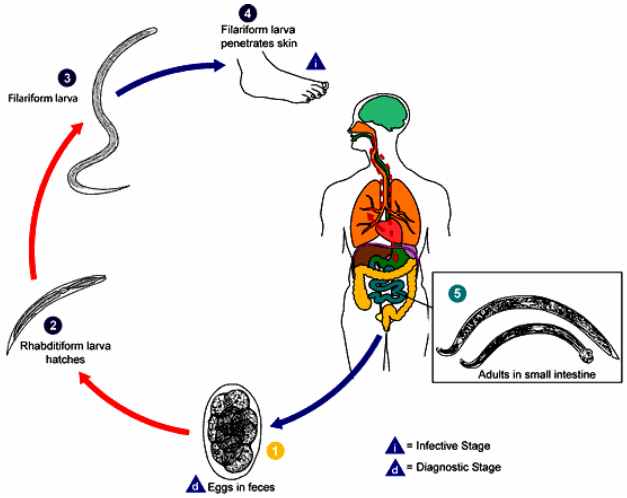
Eosinophilia occurs in about 50% patients

ANCYLOSTOMIASIS

Ancylostoma duodenale or Hookworm infection is one of the most common parasitic infections. The two Nematodes, *Ancylostoma duodenale* and *Necator americanus* cause it. Both are similar in shape and life cycle. Eggs are passed in the stool 1, and under favourable conditions (moisture, warmth, shade), larvae hatch in 1 to 2 days. The released rhabditiform larvae grow in the faeces and/or the soil 2 and after 5-10 days (and two molts) they become filariform (third-stage) larvae that are infective 3. These infective larvae can survive 3-4 weeks in favourable environmental conditions. On contact with the human host for at least 5-10 minutes, the larvae penetrate the skin and are carried through veins to the heart and lungs. They penetrate into the pulmonary alveoli, ascend the bronchial tree to the pharynx, and are swallowed 4. The larvae reach the small intestine, where they reside and mature into adults. Adult worms live in the lumen of the small intestine, where they attach to the intestinal wall with resultant blood loss by the host 5. Most adult worms are eliminated in 1-2 years, but longevity records can reach several years. Some *A. duodenale* larvae, following penetration of the host skin, can become dormant (in the intestine or muscle). In addition, infection by *A. duodenale* may probably also occur by the oral and transmammary route. Each parasite sucks about 0.1 ml of blood per day and thousands may be present in one individual. They are the most common cause of iron deficiency anaemia. Sexes are separate and both are required for production of infective fertilised ova and larvae. *Ancylostoma duodenale* has a dorsal hook that gives the parasite its name, hookworm. Both ova and larvae are passed in faeces and occasionally the adult worm may also be seen in stools. One female produces about 5000-10000 eggs/day.

LABORATORY DIAGNOSIS

1. **Ova in stools:** For morphology of ova see chapter on examination of faeces (page 93).
2. **Rhabditiform larvae:** Non-infective larvae are seen in old stools**.** Rhabditiform larvae of hookworm have snake like, purposeful movements. They have a long buccal cavity and genital primordium is insignificant.
3. **Adult parasites**: These are seen in stools after treatment.



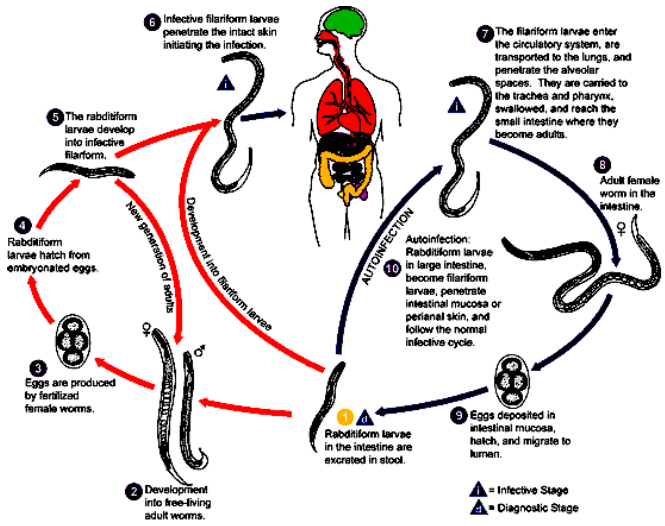
STRONGYLOIDIASIS

It is one of the 10 most common helminth infestations in the world caused by a nematode, *Strongyloides stercoralis*. It particularly occurs in warm and humid climate. It causes anaemia and hypoproteinaemia. Sexes are separate. The parasite is microscopic, the adult measuring only 1-2 mm in length and it lives in the small intestine. It has three types of life cycles:

1. **The direct cycle** is similar to hook worms except that eggs are not passed in faeces. Instead these hatch in the intestine and rhabditiform larvae (1) are passed. These transform to infective filariform larvae (6) in 2-3 days and penetrate the skin of a person.
2. In the **indirect cycle**, larvae mature on the soil into adults(2). They mate and fertilised ova are passed on soil (4). From these the rhabditiform larvae hatch which transform to filariform larvae(5). These enter the body of a human being or repeat the indirect cycle (6).
3. In **autoinfection** rhabditiform larvae (1) transform into filariform larvae inside the intestinal lumen(7). These pierce the mucosa or perianal skin and enter the blood stream to complete the tissue phase and finally reach the intestine again(8).

LABORATORY DIAGNOSIS

Rhabditiform larvae can be demonstrated in stools. Eggs are not passed except in severe diarrhoea. The larva has a short buccal cavity, a prominent genital primordium and exhibit purposeless, lashing movements as opposed to



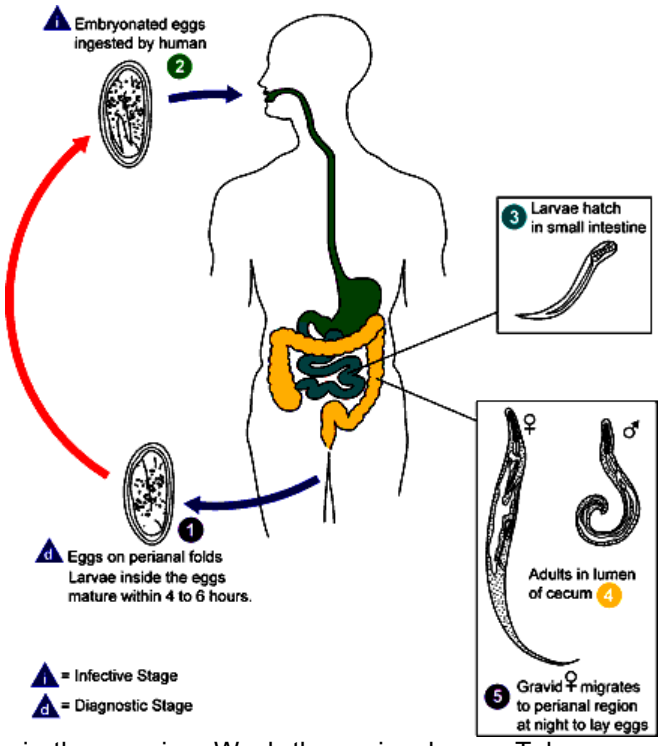
similar larvae of hookworm. If larvae are scanty in stool, they have to be concentrated by Zinc sulphate method (page 92). Occasionally larvae can be demonstrated in sputum and jejunum biopsy samples. Diagnosis can also be made by specific serological tests. The Entero-test capsule, special concentration techniques like Baermann and larval culture techniques (Harada Mori, petri dish) may also be used to yield positive results. It is important to differentiate the larvae from those of hookworms. Strongyloides filariform larvae have a slit in the tail while hookworm larvae have a pointed tail.

ENTEROBIASIS

It is one of the commonest infestations caused by a nematode, *Enterobius vermicularis* commonly called pinworm due to perianal itching and causes severe dermatitis of the perianal area. Eggs are deposited on perianal folds(1). Self-infection occurs by transferring infective eggs to the mouth with hands that have scratched the perianal area(2). Person-to-person transmission can also occur through handling of contaminated clothes or bed linens. Enterobiasis may also be acquired through surfaces in the environment that are contaminated with pinworm eggs (e.g., curtains, carpeting). Some eggs may become airborne and inhaled. The larvae hatch in the small intestine (3) and the adults establish themselves in the colon(4). The time interval from ingestion of infective eggs to production of eggs by the adult females is about one month. The life span of the adults is about two months. Gravid females migrate nocturnally outside the anus and deposit eggs there, while crawling on the skin of the perianal area(5). The larvae contained inside the eggs develop (the eggs become infective) in 4-6 hours under optimal conditions (1). Retroinfection, or the migration of newly hatched larvae from the anal skin back into the rectum, may occur. Parasites are found in the large intestine and appendix but may also migrate into the urinary bladder and female genital tract from perineum. The female is 5-10x0.5 mm in size, while male is only 2-5 mm long.

LABORATORY DIAGNOSIS

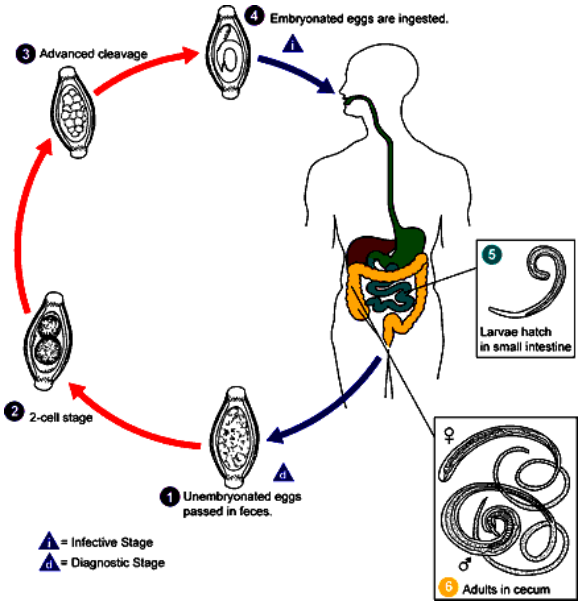
Diagnosis of pinworm infection is made on recovery of the characteristic eggs. As eggs usually are not laid inside intestine so they may not be found in stools. Gravid females may be seen in stools. These may also be seen crawling on perianal area at night (for details see page 92). Scotch tape preparation is best to demonstrate the ova of *Enterobius vermicularis*. It is important that the preparation is made early



in the morning. Wash the perianal area. Take a scotch tape and curve around one end of wooden tongue depressor with the sticky surface outside. A minimum of 6-8 consecutive negative tapes are required to rule out infection Separate the anal folds and touch all around the perianal area with the sticky surface. Spread the scotch tape on a glass slide and examine under a microscope.

TRICHURIASIS

It is caused by a nematode; *Trichuris trichiura*



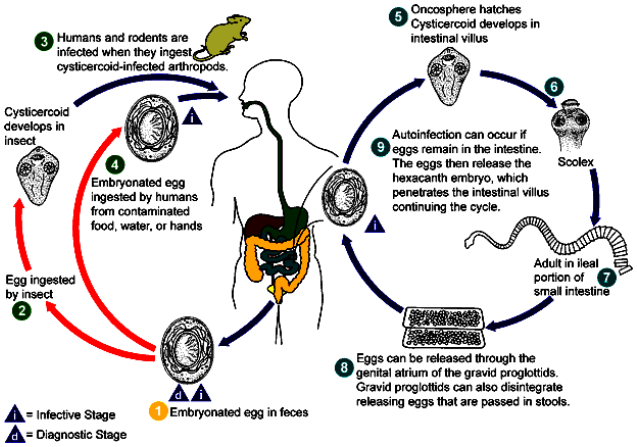
commonly called whip worm. The adult worm is 3-5 cm long with anterior 3/5 slender, is embedded in mucosa and is thread-like. Posterior 2/5 is thick and bulbous and thus resembles a whip. Posterior end of the male is coiled like a watch spring. The parasites may cause ulcerative lesions in large intestine and appendix. The gravid female lays 3000-7000 eggs daily, which take 3 weeks in soil to mature and become infectious. The unembryonated eggs are passed in stool 1. In the soil, the eggs develop into a 2-cell stage 2, an advanced cleavage stage 3, and then they embryonate 4 eggs become infective in 15 to 30 days. After ingestion (soil-contaminated hands or food), the eggs hatch in the small intestine, and release larvae 5 that mature and establish themselves as adults in the colon 6.

LABORATORY DIAGNOSIS

It is made by demonstration of characteristic barrel or football shaped eggs in the faeces measuring 50-54 µm in length, with refractile prominences (usually referred to as polar plugs) at either end. Zinc sulphate floatation method is extremely useful in demonstrating the parasites (page 90).

HYMENOLEPIASIS

It is one of the most common infestation caused by a cestode, *Hymenolepis nana* or dwarf tapeworm. It causes abdominal pain, weight loss, diarrhoea, anorexia and weakness,



malabsorption. Hypoproteinaemia with stunted growth may occur but allergic symptoms are more common. Adult worm lives in the small intestine and measures 15-25x0.5 mm. It is segmented and has a scolex. Gravid segment becomes 4 times larger. Eggs are infective when passed in stool and cannot survive more than 10 days in the external environment 1. When an arthropod intermediate host ingests eggs 2,

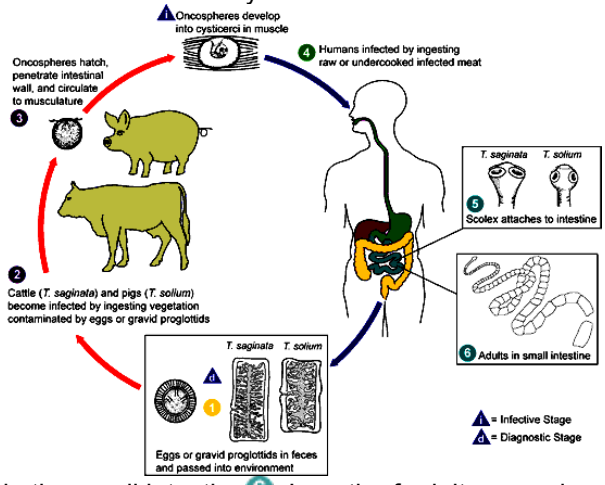
they develop into cysticercoids, which can infect humans or rodents upon ingestion 3 and develop into adults in the small intestine. When eggs are ingested 4 (in contaminated food or water or from hands contaminated with faeces), the oncospheres (hexacanth larvae) are released, penetrate the intestinal villus and develop into cysticercoid larvae 5. Upon rupture of the villus, the cysticercoids return to the intestinal lumen, evaginate their scoleces 6, attach to the intestinal mucosa and develop into adults that reside in the ileal portion of the small intestine producing gravid proglottids 7. Eggs are passed in the stool when released from proglottids through its genital atrium or when proglottids disintegrate in the small intestine 8. An alternate mode of infection consists of internal autoinfection, where the eggs release their hexacanth embryo, which penetrates the villus continuing the infective cycle without passage through the external environment 9. The life span of adult worms is 4 to 6 weeks, but internal autoinfection allows the infection to persist for years

LABORATORY DIAGNOSIS

It is made by demonstration of typical ova in faeces (page 92). Egg morphology is more easily seen in fresh specimens or those preserved in formalin based fixatives.

TAENIASIS

One of the most common parasitic infections is caused by two cestodes, *Taenia saginata* and *Taenia solium*. Their type depends upon religious beliefs. In non-pork eating persons, *Taenia solium* does not occur, as pig is the intermediate host for this. On the other hand, those who do not eat beef (Hindus) do not have *Taenia saginata* as the intermediate host is cattle. The parasite is hermaphrodite. Humans are the only definitive hosts for *Taenia saginata* and *Taenia solium*. Eggs or gravid proglottids are passed in faeces 1; the eggs can survive for days to months in the environment. Cattle (*T. saginata*) and pigs (*T. solium*) become infected by ingesting vegetation contaminated with eggs or gravid proglottids 2. In the animal's intestine, the oncospheres hatch 3, invade the intestinal wall, and migrate to the striated muscles, where they develop into cysticerci. A cysticercus can survive for several years in the animal. Humans become infected by ingesting raw or undercooked infected meat 4. In the human intestine, the cysticercus develops over 2 months into an adult tapeworm, which can survive for years. The adult tapeworms attach to the small intestine by their scolex 5 and reside



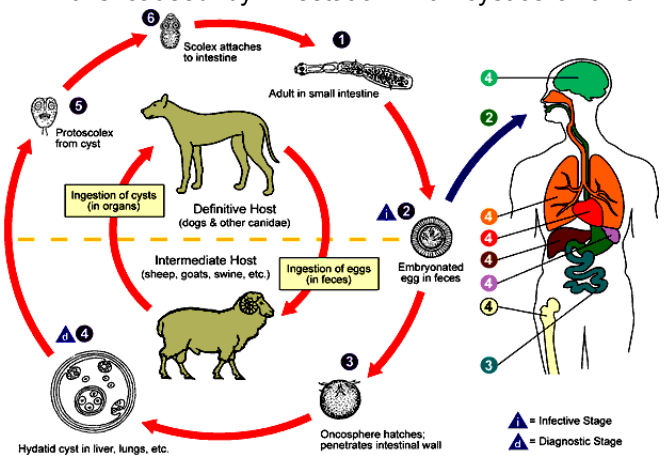
in the small intestine 6. Length of adult worms is usually 5 m or less for *T. saginata* and 2-7 m for *T. solium*. The adults produce proglottids, which mature, become gravid, detach from the tapeworm, and migrate to the anus or are passed in the stool. *T.saginata* adults usually have 1,000 to 2,000 proglottids, while *T.solium* adults have an average of 1,000 proglottids. The eggs are released after the proglottids are passed in faeces. *T.saginata* may produce up to 100,000 and *T. solium* may produce 50,000 eggs per proglottid respectively.

LABORATORY DIAGNOSIS

It is made by demonstration of typical ova in stools (on page 92). Sometimes gravid segments (proglottids) may be seen in stool. An immunoblot method for neurocysticercosis is also available

HYDATID DISEASE

It is caused by infestation with cysticerci of a



cestode *Echinococcus granulosus*. Man is

neither the definitive nor the intermediate host for this parasite but is infected accidentally. The adult *Echinococcus granulosus* (3-6 mm) ❶ resides in the small bowel of the definitive hosts, (dogs or other canines). Gravid proglottids release eggs ❷ that are passed in the faeces. After ingestion by a suitable intermediate host (sheep, goat, swine, cattle, horses, camel), the egg hatches in the small bowel and releases an oncosphere ❸ that penetrates the intestinal wall and migrates through the circulatory system into various organs, especially the liver and lungs. In these organs, the oncosphere develops into a cyst ❹ that enlarges gradually, producing protoscolices and daughter cysts that fill the cyst interior. Ingesting the cyst-containing organs of the infected intermediate host infects the definitive host. After ingestion, the protoscolices ❺ evaginate, attach to the intestinal mucosa ❻ and develop into adult stages [1] in 32-80 days. Humans become infected by ingesting eggs [2], with resulting release of oncospheres [3] in the intestine and the development of cysts [4] in various organs.

LABORATORY DIAGNOSIS

The diagnosis of a cyst is made clinically or by x-ray, ultrasound, CT scan etc. Sometimes help is sought from laboratory. Casonis skin test is now obsolete. Latex agglutination, indirect haemagglutination, complement fixation test and arc-5 double diffusion assay are now available to detect antibody against *Echinococcus granulosus.* Microscopic examination of cyst wall and aspirated fluid for scoleces is required after removal.

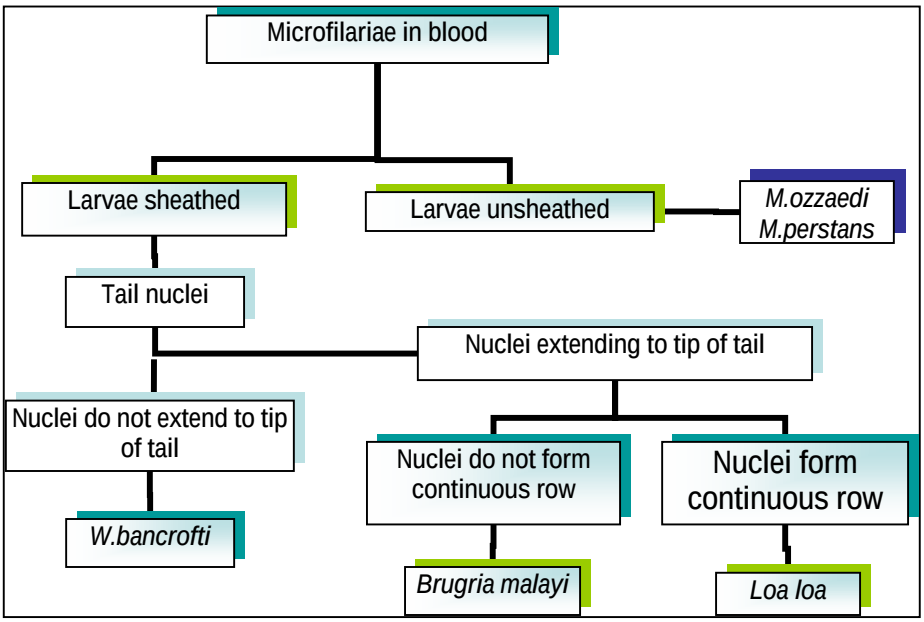


Figure 14.4. Differential diagnosis of microfilareae in blood.

SECTION IV – MICROBIOLOGY

15. CLASSIFICATION OF BACTERIA

Microorganisms are very small microscopic structures that are capable of free living. Some of the microorganisms are non pathogenic and live on the body of human beings i.e. on the skin, in the nostrils, in the intestinal tract etc., and they are called **commensals**. The organisms that are capable of causing disease are called **pathogenic** organisms. There are two groups depending upon the structure of cells:

1. Prokaryotes
2. Eukaryotes

Prokaryotes: This group includes those organisms that have a very simple cell structure and nuclear material is in the form of single chromosome but is not surrounded by a nuclear membrane. They divide by simple binary fission. Examples are bacteria, Mycoplasma, chlamydia and rickettsiae.

Eukaryotes: These organisms have complete cell structure similar to the higher organisms. The nuclear material is bounded by a nuclear membrane to form a nucleus. They have more than one chromosome, complete enzyme systems of their own and divide by mitosis. Examples are fungi and protozoa (Figure 15.1).

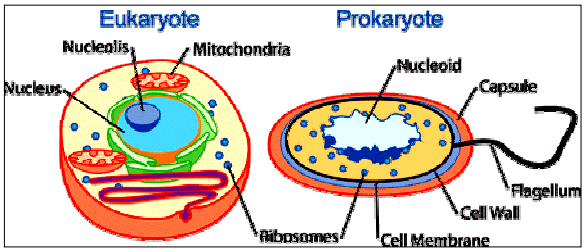


Figure 15.1: Structure of Eukaryote and Prokaryote cells

CLASSIFICATION OF BACTERIA

Bacteria can be classified depending upon:

- Morphology
- Gram staining
- Requirement for oxygen
- DNA homology

1. MORPHOLOGICAL CLASSIFICATION

On the basis of morphology bacteria are divided into the following groups:

a. Cocci: round or oval in shape
b. Bacilli: rod shaped
c. Vibrios: coma shaped
d. Spirochaetes: spiral like

Intermediate shapes like cocco-bacilli also exist.

2. CLASSIFICATION BASED ON GRAM STAINING

Bacteria are divided into Gram-negative and Gram-positive on the basis of their cell wall structure (Figure 15.2).

a. Gram-positive: Bacteria staining purple in Gram stained smear. They have thick layer of peptidoglycan.
b. Gram-negative: Bacteria staining pink in Gram stained smear. Gram-positive bacteria, when dead may stain red. They have thick outer membrane.
c. Gram variable: The organism is Gram-positive but appears Gram-negative or is Gram-negative but appears Gram-positive.

3. CLASSIFICATION BASED ON OXYGEN REQUIREMENT

a. Strict Aerobes: These do not grow in the absence of oxygen.
b. Anaerobes: These can be of two types:
   i) Strict (obligatory) anaerobes: Bacteria that can grow only in the absence of oxygen.
   ii) Facultative anaerobes: These can grow both in presence or absence of oxygen. Most of the commonly isolated bacteria belong to this group.
c. Carboxyphilic: These require presence of high percentage (10%) of carbon dioxide.
d. Microaerophilic: These require only small amounts of oxygen for their growth and higher concentration of the oxygen will kill the organism.

4. CLASSIFICATION BASED ON TEMPERATURE REQUIREMENT

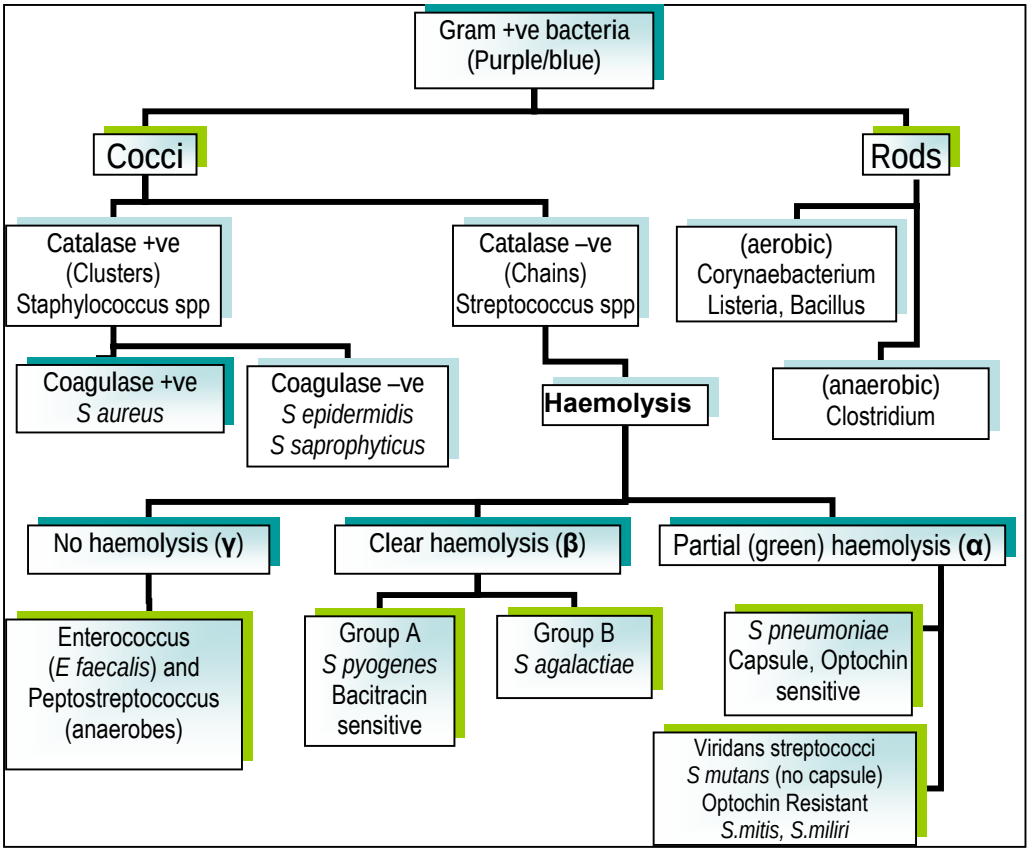
Based on the temperature requirement for their growth bacteria are classified into following three groups:

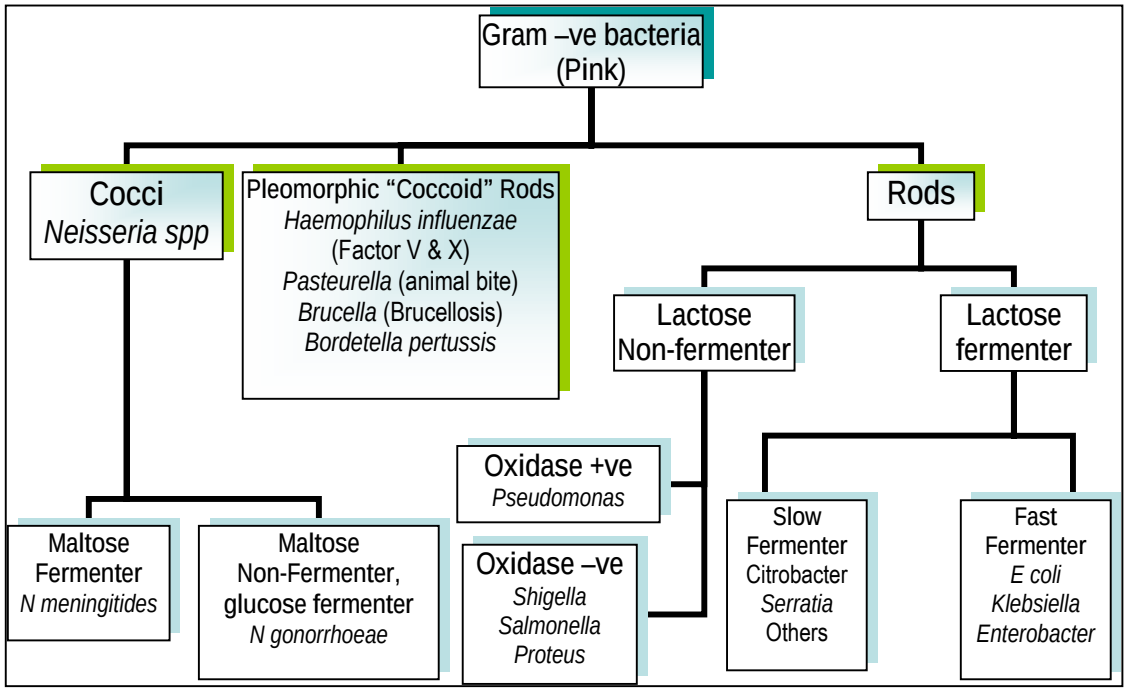
a. Mesophilic
b. Psychrophilic, and
c. Thermophilic

IMPORTANT GROUPS OF BACTERIA

1. **Gram-positive cocci**
   a. Aerobes (facultative anaerobes)
      i) Staphylococcus species
      ii) Streptococcus species
      iii) *Enterococcus species*
   b. Anaerobes (obligatory)
      i) *Peptococcus* species
      ii) *Peptostreptococcus* species
      iii) *Ruminococcus* species
2. **Gram-positive rods (bacilli)**
   a. Aerobes (facultative anaerobes)
      i) Corynebacterium species
      ii) Bacillus species
      iii) Listeria species
      iv) Lactobacillus species
      v) Nocardia species
   b. Anaerobes (obligatory)
      i) *Clostridium* species
3. **Gram-negative cocci**
   a. Aerobes (facultative anaerobes)
      i) *Neisseria* species
      ii) *Moraxella* species
   b. Anaerobes (obligatory)
      i) *Veillonella* species
4. **Gram-negative rods (bacilli)**
   a. Aerobes (facultative anaerobes)
      i) Escherichia coli
      ii) *Klebsiella* species
      iii) *Proteus* species
      iv) *Shigella* species
      v) *Salmonella* species
      vi) Vibrio species
5. **Gram-negative cocco-bacilli**
   a. Aerobes (facultative anaerobes)
      i) *Haemophilus* species
      ii) *Bordetella* species
      iii) *Brucella* species
      iv) *Legionella* species
      v) Francisella species
   b. Strict aerobes
      i) *Aeromonas* species
      ii) *Plesiomonas* species
      iii) Mycobacterium tuberculosis
      iv) *Pseudomonas* species
   c. Anaerobe (obligatory)
      i) *Bacteroides* species
      ii) *Fusobacterium* species
      iii) *Prevotella* species
   d. Microaerophilic
      i) *Campylobacter* species
      ii) *Helicobacter* pylori
6. **Spirochaetes**
   a. Aerobic
      i) *Leptospira* species
   b. Microaerophilic
      i) *Treponema* species
      ii) *Borrelia* species
7. **Intracellular Organisms**
   a. Bartonella bacilliformis
   b. *Chlamydia* species
   c. *Rickettsia* species
8. **Cell Wall Deficient Organisms**
   a. *Mycoplasma* species
   b. 'L' forms of bacteria

Figure 15.2: Flow chart for preliminary identification of bacteria





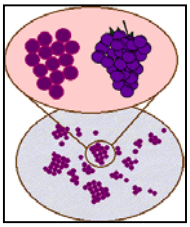
16. COCCI

GRAM POSITIVE COCCI

STAPHYLOCOCCUS

Staphylococci are common organisms found in the environment. They are present on the skin and in the anterior nostrils as commensals. Important pathogenic species are:

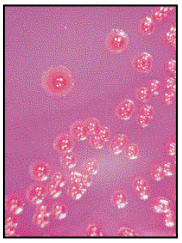
- *Staphylococcus aureus*
- *Staphylococcus epidermidis*
- *Staphylococcus saprophyticus*



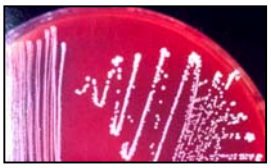
MORPHOLOGY

They are Gram-positive cocci, 0.5-1 µm in diameter, arranged in irregular clusters, singly or in pairs.

CULTURAL CHARACTERISTICS



They are facultative anaerobes but grow best in aerobic environment at 35-37°C on blood agar, MacConkey agar and mannitol salt agar as a selective medium. This selective medium is specially used in cases of food poisoning caused by staphylococci. *S. aureus* colonies are about 1-2 mm in size and yellow to golden in colour. A zone of complete haemolysis can usually be seen when cultured on blood agar. *S.epidermidis* colonies are white and usually do not produce haemolysis.



ENZYMES OF *STAPHYLOCOCCUS AUREUS*

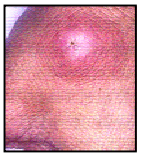
1. Catalase: converts H_2O_2 to H_2O and O_2
2. Coagulase: converts fibrinogen to fibrin.
3. DNAse: splits deoxyribonucleic acid (DNA)
4. Phosphatase: breaks phosphates.
5. Lipase: breaks fats
6. Hyaluronidase: splits hyaluronic acid
7. Staphylokinase causes fibrinolysis.
8. β-lactamase: breaks down the penicillin by attacking its structural ring.

TOXINS OF *STAPHYLOCOCCUS AUREUS*

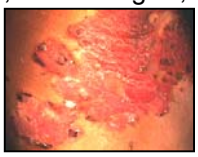
1. Haemolysins, α, β, γ and θ
2. Toxic shock syndrome toxin (TSS)
3. Exfoliative toxin causes peeling of skin and scalded skin syndrome.
4. Leucocidin (Panton-Valentine [P-V] substance) kills white blood cells.
5. Enterotoxin (A-F) causes food poisoning.

PATHOGENICITY

The pathogenic species is *S. aureus*. It causes:



1. Boils, abscesses furuncles and carbuncles
2. Wound infections
3. Hospital infections
4. Conjunctivitis
5. Pneumonia, osteomyelitis, meningitis, endocarditis



6. Food poisoning
7. Scalded skin syndrome in children
8. Toxic shock syndrome

S. epidermidis is a normal commensal but may cause endocarditis especially in prosthetic valves, ventricular shunts and in drug addicts. This organism is also an important cause for intravascular catheter associated blood stream and other infections particularly in immunocompromised patients. *S. saprophyticus* causes urinary tract infection in females. Both are coagulase and DNAse negative and can be differentiated by putting up the antimicrobial sensitivity disk of Novobiocin or colistin (Polymyxin). *S.saprophyticus* is resistant to Novobiocin and susceptible to colistin whereas *S. epidermidis* is susceptible to Novobiocin and resistant to colistin.

Table 16.1: biochemical reactions of *staphylococcus aureus*

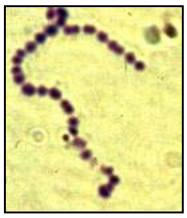
Test	Reaction
Catalase	+ve
Coagulase	+ve
DNAse	+ve
Phosphatase	+ve
Mannitol fermentation	+ve
VP	+ve

ANTIBIOTIC SENSITIVITY

Antibiotic disks employed in sensitivity testing of *Staphylococci* are Penicillin (>90% *Staphylococci* are penicillin resistant), Oxacillin,

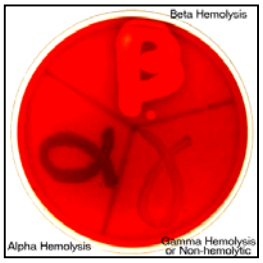
Erythromycin, Tetracycline, Cephalosporins (1st generation), Lincomycin, Clindamycin, Fusidic acid, Vancomycin, Teicoplanin, Gentamicin, Amikacin, Quinolones, Rifampicin. The susceptibility against cloxacillin is NOT tested with the disk of cloxacillin, instead the disk of oxacillin (1 µg) is used. If *Staphylococcus aureus* is resistant to oxacillin it is labelled as methicillin resistant and known as **MRSA** (**Methicillin resistant *Staphylococcus aureus***). MRSA shows multi-resistance to antibiotics and is invariably resistant to other β-lactam antibiotics (cephalosporins, imipenem etc.). They are very important hospital pathogens and are extremely difficult to eradicate.

STREPTOCOCCUS



They are Gram-positive cocci arranged in chains of varying lengths. Some are seen in pairs. Colonies are small (0.5 mm), matt and grey-white in colour. They are classified on the bases of haemolysis on sheep blood agar plate into three groups.

1. β-haemolytic *Streptococci*. There is a zone of complete haemolysis around colonies.
2. α-haemolytic *Streptococci*. There is incomplete zone of haemolysis around the colonies shown as green discolouration.
3. Nonhaemolytic (γ). No haemolysis at all.



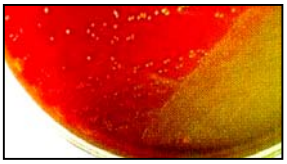
β-haemolytic *Streptococci* are further classified on the basis of the presence of group specific carbohydrate (C-polysaccharide) in the cell wall into groups from A to H and K to V (**Lancefield grouping**). The carbohydrate antigen is extracted from bacterial suspension by enzymes, heat or acid, which can then be identified by agglutination reaction with a drop of specific antiserum.

IMPORTANT SPECIES

1. *Streptococcus pyogenes* (Group A) β-haemolytic
2. *Streptococcus agalactiae* (Group B) β-haemolytic
3. *Enterococcus* and non-enterococcus species (Group D), α-or nonhaemolytic or β-haemolytic on sheep blood agar
4. *Streptococcus viridans*, α-haemolytic
5. *Streptococcus pneumoniae*, α-haemolytic

HABITAT/METABOLISM

Streptococci are catalase negative and facultative anaerobes, grow best at 35-37°C and need enriched media like blood agar. They do not grow on MacConkey agar except, enterococci (*E. faecalis)* and some strains of group-B (*S. agalactiae*).



ENZYMES

1. Streptokinase (Group A-C) virulence factor, lysis of clot/fibrin.
2. Hyaluronidase – spreading factor
3. DNAse/Ribonuclease
4. Diphosphopyridine nucleotidase

TOXINS

1. Streptolysin-S (haemolysis on aerobic plate), oxygen stable
2. Streptolysin-O (haemolysis on anaerobic plate), oxygen labile, cardiotoxic.
3. Erythrogenic toxin (rash in scarlet fever), pyrogenic toxin.
4. Leucocidin
5. M-protein, major virulence factor (Group A)

PATHOGENICITY

Streptococcus pyogenes (Group A) is the most important pathogen. It causes:

1. Sore throat, tonsillitis, pharyngitis and peritonsillar abscess
2. Puerperal sepsis
3. Ear infections
4. Skin infections (erysipelas)
5. Scarlet fever (fever with rash)
6. Septicaemia and endocarditis

Post-streptococcal infection diseases (Nonsuppurative complications)

Rheumatic fever, and acute glomerulonephritis are two diseases, which are not due to direct invasion of the organisms but because of immune response of the body to the bacterial antigens. These diseases usually appear 3-4 weeks after the streptococcal infection. Acute glomerulonephritis occurs after streptococcal skin infection. The kidneys are affected and RBCs and albumin are passed in the urine. Rheumatic fever occurs after streptococcal sore throat in which heart valves are damaged and big joints are affected. Permanent damage of the heart valves occurs. It is a childhood disease.

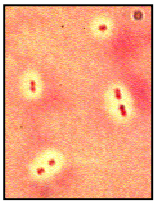
Other diseases

1. *S.agalactiae* (Group B):
   Neonatal septicaemia
   Neonatal pneumonia
   Neonatal meningitis
   Puerperal sepsis
   Septic abortions
2. Enterococci (Group-D)
   Urinary tract infections
   Septicaemia
   Endocarditis
   Wound infections
3. *S.viridans* (nongroupable)
   Dental caries, endocarditis in patients who have artificial heart valves or damaged heart valves by disease.
4. Group C and G streptococci
   Nasopharynx of healthy persons

ANTIBIOTIC SENSITIVITY

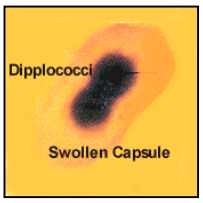
S. pyogenes is almost always and *S. agalactiae* usually sensitive to penicillin. They may also be sensitive to erythromycin, quinolones, minocyclines, rifampicin, clindamycin, vancomycin and teicoplanin. *Enterococcus faecalis* is usually sensitive to ampicillin but not to Benzyl penicillin. Enterococci are usually resistant to gentamicin but in cases of endocarditis caused by these organisms combination of ampicillin and gentamicin is effective. However, if gentamicin is to be prescribed, its susceptibility should be checked with high content disk (120 µg) of gentamicin instead of usual disk containing 10 or 30 µg. Combination of ampicillin and gentamicin is recommended if the organism is found susceptible to high content disk of gentamicin. Enterococci are common pathogens in hospital acquired, infections and may be resistant to most of the antibiotics available and only choice remains is vancomycin or teicoplanin. However, there are reports of resistance against these antibiotics also, known as vancomycin resistant enterococci (VRE).

STREPTOCOCCUS PNEUMONIAE



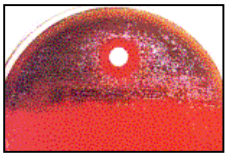
Streptococcus pneumoniae can be found in the upper respiratory tract as a commensal. They are Gram-positive lancet shaped (lanceolate) diplococci in pairs with their long axis in line. They are easily decolourised and hence are usually seen as Gram-negative in sputum or on slides made from culture. Virulent pneumococci are capsulated and form mucoid, smooth colonies.

CULTURAL CHARACTERISTICS



Optimum growth requires enriched media and grow best on chocolate agar with 5-10% CO_2 (candle jar). Colonies are usually very small, circular and raised. Later they become flattened in the centre given the name, Draughtsman colonies. There is often a zone of α (incomplete) haemolysis around the colonies on blood agar.

IDENTIFICATION



They can be differentiated from *S. viridans* by their optochin sensitivity, bile solubility, fermentation of inulin and pathogenicity in mice.

PATHOGENICITY

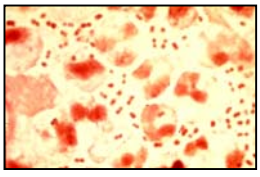
They cause lobar pneumonia, broncho-pneumonia, meningitis, ear infection, arthritis, pericarditis, sinusitis and septicaemia etc.

ANTIBIOTIC SENSITIVITY

Most strains are penicillin sensitive but some strains are penicillin resistant. To determine the susceptibility against penicillin, antimicrobial disk of penicillin is not used but the disk of Oxacillin (1 µg) is used. This procedure will detect not only strains resistant to penicillin but also relative resistant strains. The relatively resistant strains can be treated with penicillin but they require higher therapeutic dose. Other drugs used against these organisms are erythromycin, clarithomycin, cephalosporins, newer quinolones etc. Penicillin and cephalosporin resistant strains are a major therapeutic problem, especially while treating acute suppurative meningitis.

GRAM NEGATIVE COCCI

NEISSERIA

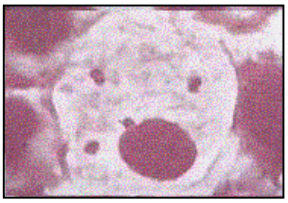


These are Gram-negative diplococci. Non-pathogenic species are sometimes present as commensal in the upper respiratory tract. Important pathogenic species are *Neisseria meningitidis* and *Neisseria gonorrhoeae.*

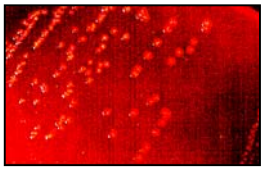
MORPHOLOGY

They are Gram-negative, kidney shaped cocci 0.5-1 µm in size usually arranged in pairs with

their long axis parallel and flattened sides facing one another. They are usually found inside the pus cells. Both *Neisseria* species are capsulated.



GROWTH CHARACTERISTICS



Pathogenic Neisseriae are aerobic, require enriched media with 5-10% CO_2. Commonly used media are blood agar, chocolate agar, Modified New York City medium and Thayer and Martin agar (Selective with antibiotics). On Chocolate agar colonies are small, 1-2 mm in diameter, shiny and grey in colour.

Table 16.2: Biochemical reactions of Neisseria.

Species	Glucose	Maltose	Sucrose	Lactose	Starch
N.meningitidis	+	+	-	-	-
N.gonorrhoeae	+	-	-	-	-
Moraxella catarrhalis	-	-	-	-	-
N.sicca	+	+	+	-	+
N lactamica	+	+	-	+	-

BIOCHEMICAL REACTIONS

All Neisseriae are oxidase positive. Sugar fermentation reactions (Table 16.2) are used to differentiate between *N.meningitidis*, *N.gonorrhoeae* and *Moraxella catarrhalis* (Previously *Branhamella catarrhalis*) and non-pathogenic Neisseriae spp. Sugar sets must be prepared in Hiss's serum.

PATHOGENICITY

Neisseria meningitides

Neisseria meningitidis has several serological groups based on capsular polysaccharides (A, B, C, X,Y, W135). It is the etiological agent of meningococcal meningitis, infection of the meninges (membranes covering the brain). Meningococcal meningitis often occurs in epidemics among young adults and outbreaks among military recruits (mostly due to Group A & C strains). Organisms enter through nasopharynx. From there they reach the blood stream (meningococcaemia) and then infect the meninges.

Neisseria gonorrhoeae

It causes gonorrhoea, a sexually transmitted disease (STD). Gonococci attack the mucous membrane of the genital tract, rectum, eyes and rarely throat. They produce inflammation that may become chronic and cause fibrosis. In men, the urethra is infected causing a purulent discharge. In chronic cases it may lead to urethral strictures. In females, cervix is infected and the infection can spread into fallopian tubes via the uterus and hence may lead to pelvic inflammatory disease and infertility. Gonococcal arthritis (usually of knee joints) is common in disseminated infection.

ANTIBIOTIC SENSITIVITY

N.meningitidis is invariably susceptible to penicillin but most of the strains are resistant to sulphonamides. The other antibiotics which can be used are fluoroquinolones (ciprofloxacin, ofloxacin etc.), rifampicin, third generation cephalosporins (like ceftriaxone) and chloramphenicol. Penicillin, tetracycline, streptomycin, ceftriaxone and spectinomycin are used for *N.gonorrhoeae*. There are several reports of penicillin resistance in *N. gonorrhoeae* due to penicillinase (β-lactamase) production.

17. BACILLI

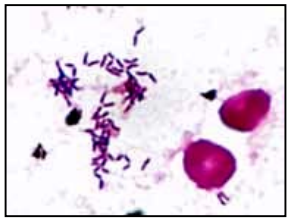
GRAM POSITIVE BACILLI

CORYNEBACTERIUM

Corynebacterium species are widely distributed in nature. Many are part of normal flora of the skin, nasopharynx, oropharynx, urogenital and intestinal tract. *Corynebacterium diphtheriae* causes diphtheria. Other species generally known as diphtheroids and include:

1. *Corynebacterium ulcerans*
2. *Corynebacterium hoffmani*
3. *Corynebacterium xerosis*

MORPHOLOGY



They are small, Gram-positive, pleomorphic rods arranged at angles to each another (Chinese letters arrangement) and show irregular staining. In **Albert stained smear**, the rods are green in colour containing granules at the ends or in the centre that stain purple blue. These are called metachromatic or **volutin** granules. On the basis of pleomorphism and arrangement, one can differentiate *Corynebacterium diphtheriae* from other corynebacteria.

CULTURAL CHARACTERISTICS

Corynebacteria are aerobic and facultative anaerobes. Optimum growth temperature is between 35-37°C. They require enriched media for their growth. Commonly used media are blood agar, tellurite blood agar (selective medium), Modified Tinsdale Medium (selective and differential medium) and Loeffler's serum medium. On blood agar the colonies are small, 1-2 mm, mucoid, haemolytic or non-haemolytic. On tellurite blood agar colonies are grey to black in colour. On modified Tinsdale Medium black colonies of *Corynebacterium diphtheriae* have a brown halo around them whereas, diphtheroids do not have a halo. Growth on Loeffler's serum is rapid (4-6 hours) and morphology is better appreciated. Moreover, the toxin production is superior and can be used for animal inoculation. *Corynebacterium diphtheriae* has three biotypes, i.e. gravis, mitis and intermedius. One can differentiate them from their colonial morphology and haemolysis on blood agar.

BIOCHEMICAL IDENTIFICATION

Corynebacterium diphtheriae biotypes can be distinguished by fermentation of Hiss serum sugars. The reactions are shown in Table 17.1.

Table 17.1: Biochemical reactions of Corynebacteria.

Species	Glucose	Maltose	Sucrose	Starch	Dextrin
C.diphtheriae gravis	+	+	-	+	+
C.diphtheriae mitis	+	+	-	-	-
C.diphtheriae intermedius	+	+	-	-	-

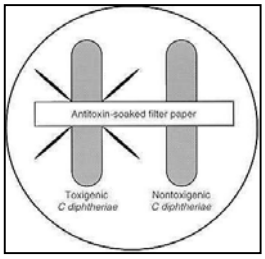
EPIDEMIOLOGY AND CLINICAL DISEASES

Human disease is caused by droplet dissemination, or direct contact with cutaneous carries. Usually pharyngeal and cutaneous forms of the disease are seen. The mortality is due to the effects of **exotoxin**.

DEMONSTRATION OF TOXIN

Only toxin-producing strains of *Corynebacterium diphtheriae*, are capable of causing disease, therefore, demonstration of toxin production is necessary in the laboratory. Following methods can be utilised:

a. Agar gel diffusion (precipitin test, ELEK'S Plate)
b. Animal inoculation
c. PCR



ELEK'S plate is made from horse serum agar. A filter paper strip soaked in diphtheria antitoxin is placed on the surface of the medium in the middle. The test, positive and negative control organisms are inoculated in the form of streaks at right angles to the strip, taking care not to touch the strip and incubated aerobically at 37°C overnight (to be continued for 4 days if the results are negative). The plate is examined for the lines of precipitation against a dark background. A line similar to positive control is formed if the organism is toxigenic. Animal inoculation test is done either by subcutaneous or intradermal injection of the suspension of the organisms into guinea pig. Two guinea pigs are used, one is protected by antitoxin. In subcutaneous injection the unprotected animal dies whereas in

intradermal injection the skin of unprotected animal shows erythema and necrosis due to the effect of toxin.

PATHOGENICITY

Since the introduction of mass immunisation, the incidence of diphtheria has markedly reduced. In children the organism infects the mucous membrane of tonsils, pharynx and upper respiratory tract. During their multiplication the organisms produce an exotoxin that causes necrosis of the mucous membranes and there is pseudomembrane formation. This toxin is also absorbed in blood and has its effects on heart and nerves. If the diphtheritic membrane extends down into the larynx (laryngeal diphtheria), it can obstruct the airway and cause death.

Schick test
This is a skin test to demonstrate the presence of immunity against diphtheria. For details see SCHICK TEST on page 231.

ANTIBIOTIC SENSITIVITY

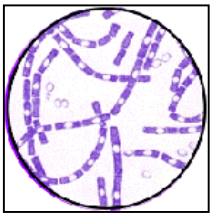
Antimicrobial therapy is not helpful, since organism is non-invasive. All patients must be given antitoxin immediately. *Corynebacterium diphtheriae* is sensitive to penicillin and erythromycin but diphtheroids are usually resistant. In fact these organisms are resistant to most of the antibiotics. Vancomycin remains the only choice.

BACILLUS SP

They are large, Gram-positive rods, commonly present in soil, dust and water. Species of medical importance are *Bacillus anthracis*, which causes anthrax (malignant pustule), and *Bacillus cereus*. *B.cereus* is motile, completely haemolysing sheep blood and is susceptible to gamma phage and causes food poisoning.

MORPHOLOGY

They are Gram-positive rods with square ends, arranged in chains. Some of them produce endospores, seen as unstained areas in the Gram stain. Spores are stained with special methods. The capsule is made up of proteins, containing D-glutamic acid. It stains purple with polychrome methylene blue stain, known as Macfaydean's Reaction (for details see page 163). All species of *Bacillus* are motile except *B. anthracis*.



CULTURAL CHARACTERISTICS

Bacillus anthracis is highly infectious and must be handled with great care in safety cabinet. The organism is aerobic, grows best at 36-37°C but spore formation is best seen at 25-30°C. Commonly used media for its isolation are blood agar and mannitol, Egg Yolk, Phenol red, Polymyxin agar (MYPA). Colonies on blood agar are large, grey-white, 2-5 mm in size, raised with wavy edges, mucoid and usually non-haemolytic. Saprophytic *Bacillus* species are usually haemolytic.

IDENTIFICATION

It liquifies gelatin but this is slow to develop. The characteristics helpful for preliminary identification are Gram-positive rods, non-haemolytic colonies on sheep blood agar, lack of motility and positivity for Macfaydean's stain (page 163). Further identification is done by biochemical tests, immunofluorescence, animal inoculation and by determination of specific plasmids by PCR. A Guinea pig inoculated with culture growth or material from the pustule dies within 48 hours.

PATHOGENICITY

Anthrax is a disease of cattle and horses. They are infected by ingestion of spores. Humans can get infection by; 1) introduction of spores into broken skin when in contact with infected animal, skin or wool (cutaneous anthrax), 2) by inhalation of spores (pulmonary anthrax) and 3) by ingestion of spores (gastrointestinal anthrax). Spores germinate and cause gelatinous oedema and congestion. Bacilli may go into blood and cause septicaemia, meningitis, haemorrhagic pneumonia and shock.

ANTIBIOTIC SENSITIVITY

Bacillus species are sensitive to penicillin, tetracycline, streptomycin, cotrimoxazole and fluoroquinolones (ciprofloxacin and ofloxacin).

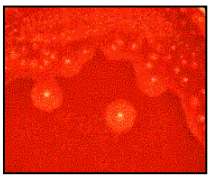
CLOSTRIDIA

Clostridia are Gram-positive spore forming rods. The important species are *Clostridium perfringens, Clostridium tetani*, *Clostridium botulinum* and *Clostridium difficile*. *Clostridia* are found in dust contaminated with horse and cattle dung and in the intestinal tract of human and animals.

MORPHOLOGY

They are Gram-positive, spore forming, rods. Spores can be terminal, sub-terminal or central and bulge out from the width of bacilli. Spores of *Clostridium perfringens* are only found in organisms growing in intestinal tracts and not in artificial culture media. All clostridia are motile except *Clostridium perfringens.*

CULTURAL CHARACTERISTICS



They only grow in anaerobic environment. The optimum temperature for growth is 35-37°C. Agar containing culture media can be placed in anaerobic jars. Fluid media that contain reducing materials e.g., Robertson's cooked meat (RCM), thioglycollate medium and media containing iron nails can be used. Colonies are shown in

CLOSTRIDIUM PERFRINGENS

It causes gas gangrene or anaerobic myonecrosis. There are 6 serotypes, from A-F. All strains produce α-toxin (lecithinase). Only type A, C and F produce the disease. Colonies are large, round and smooth with a zone of haemolysis. Spores are formed under natural conditions, only rarely in cultures. Spores resist the routine antisepsis. They are moderately heat resistant but the food poisoning strains are more heat resistant.

Metabolic activity

C. perfringens is active biochemically, fermenting glucose, maltose, lactose and sucrose. H_2S gas, proteolytic enzymes, gelatinase are produced.

Pathogenicity

The pathogenicity of *Clostridium perfringens* is because of α-toxin produced by all strains. This is an enzyme called lecithinase C. As lecithin is present in all the cell membranes so it attacks all the cells and causes lysis (and haemolysis of RBCs). It has a lethal, generalised necrotising action. Other toxins produced include β-toxin, δ-toxin and enterotoxin responsible for food poisoning.

Identification

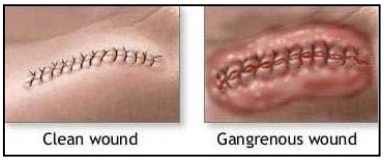
On neomycin blood agar a disk of metronidazole is placed. Wilkins Chalgren agar can be used for rapid identification. Anaerobes are genetically resistant to aminoglycosides but most of them are sensitive to metronidazole. Hence, clostridia grow on neomycin blood agar plate but there is a zone of sensitivity around metronidazole.

Reaction in Cooked Meat Medium: *Clostridium perfringens* is saccharolytic i.e., it breaks down carbohydrates and produces reddening of the meat in the medium, which develops bad smell. Gas is also produced.

Nagler's Reaction: Lecithinase producing bacteria cause opalescence in human serum or egg yolk media as demonstrated in Nagler's reaction. A petri-dish containing egg yolk medium is covered on half of its surface by α-antitoxin. The test and control organisms are inoculated in the form of streaks taking care to start the inoculation from the side where there is no antitoxin. The plate is incubated anaerobically at 37°C overnight. The lecithinase-producing organisms will show opalescence of medium around the growth but this opalescence will be absent on the side having antitoxins due to neutralisation of lecithinase by specific antitoxin. *Clostridium perfringens* gives a positive reaction.

Litmus Milk Test: A clot is formed in the medium by the gas produced in it and is called stormy clot reaction. Lactose Egg Yolk Milk Agar is a differential medium for anaerobes. Following reactions may be seen in the medium:

1. Lecithinase activity seen as opalescence in the medium.
2. Lipolysis seen as pearly layer covering the colonies.
3. Lactose fermentation seen as red colonies on exposure to air.
4. Proteolysis visible as clearing around the colonies.



Pathogenicity

Clostridium perfringens produces **gas gangrene** in association with *Cl.oedematiens, Cl.septicum and Cl.histolyticum.* There is swelling (oedema) of the tissue, infection of muscles and their necrosis and foul-smelling gas production. This occurs if a wound is infected with spores of *Cl.perfringens* e.g., in a roadside accident or introduction of dust contaminated with animal manure (khaad). **Food poisoning** is produced by more heat resistant, type A strains, which are non-haemolytic. It is caused by an exotoxin, liberated in the gut by contaminated food. It is characterised by nausea, abdominal pain and diarrhoea.

CLOSTRIDIUM TETANI

It is a slender, Gram-positive, spore forming, motile, strict anaerobe bacillus. The spores are spherical and terminal, giving a drumstick

appearance. The organism causes **tetanus** due to the production of a neurotoxin (tetanospasmin) and tetanolysin, which causes lysis of RBC. Tetanospasmin effects the central nervous system and causes muscle spasm. *Cl.tetani* spores are introduced in the wounds where they germinate and produce toxin that enters the blood and produces its effect on CNS. Tetanus can be prevented by tetanus toxoid, and immunisation of pregnant women prevents tetanus in neonates. Unlike gas gangrene, the toxin does not affect the local wound. The wounds are necrotic and soiled with dust. Infants get tetanus by infection of umbilical cord. *Cl.tetani* are grown on blood agar in the form of fine layer covering the surface, which may easily be overlooked. Haemolysis is usually present on horse blood agar. The organisms are difficult to isolate. On RCM there is blackening of the meat, as the organism is weakly proteolytic.

Prevention
Tetanus can be prevented by immunisation with tetanus toxoid. Tetanus in neonates can be prevented by immunisation of the pregnant women.

CLOSTRIDIUM BOTULINUM

C.botulinum produces the most potent poison known. Five types, from A to E, cause **botulism**, a severe and fatal form of food poisoning due to toxin produced in the contaminated tinned or other food. The ingestion of pre-formed toxin in food results in food poisoning. The toxin causes paralysis of the muscle leading to death due to respiratory failure. For diagnosis, food, faeces and vomitus are tested for toxin. On blood agar the colonies are large with a wavy outline and granular surface. Haemolysis is usually present on horse blood agar. On RCM various strains have different reactions. Organism is weakly proteolytic, produce gelatinase and H_2S, and is lipase positive.

CLOSTRIDIUM DIFFICILE

It is associated with antibiotic-induced pseudomembranous colitis. During broad-spectrum antibiotic therapy, resistant strains of *C.difficile* overgrow and produce exotoxins. Oral vancomycin is usually given for treatment.

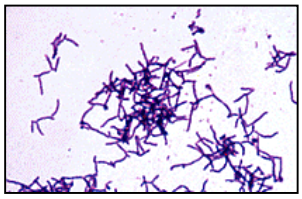
ACTINOMYCETES

These are Gram-positive, filamentous bacilli, microscopically resembling mycobacteria but superficially resembling fungi. They grow as branching filaments, which tend to break down into bacteria like pieces. These are either:

1. Aerobes:
   a. Actinomadura species
   b. Nocardia species
   c. Streptomyces species, or
2. Anaerobes:
   a. *Actinomyces* species other than maedura.

The anaerobic species are present in the mouth as part of the normal flora. *Nocardia* is the only genus, which is acid fast to 1% sulphuric acid in modified Ziehl Neelsen Staining.

ACTINOMYCES



Actinomyces israelii causes chronic suppurative infection of the Cervico-facial region, chest, or right iliac fossa of abdomen. The pus contains sulphur granules, which are actually the colonies of these organisms. When the granules in the pus are washed in distilled water, crushed and stained with Gram stain, typical morphology of the organism is seen. In the centre branching, filamentous, Gram-positive bacteria are present, surrounded by star shaped Gram-negative forms. In modified Ziehl-Neelsen stain (decolourisation with 1% H_2SO_4) the branches are non-acid fast but the peripheral clubs are acid fast.

Cultural Characteristics
The organisms grow on blood agar but require anaerobic or microaerophilic incubation at 37°C. The growth appears in 4-7 days. The colonies are creamy or white-grey in colour with an irregular surface resembling the surface of a tooth (dentate) and are adherent to the medium.

Biochemical Reactions
This organism is positive for catalase, indole and hydrolysis of aesculin but is urease negative.

Antimicrobial Sensitivity
They are sensitive to penicillin, clindamycin and tetracyclines.

NOCARDIA

Important species are *Nocardia brasiliensis* and *Nocardia asteroides*. They cause mycetoma (**Madura foo**t), lung abscess and at times brain abscess. They are Gram-positive, with bacillary and coccoid forms, aerobic and partially acid fast (1% acid). Specimens include pus, sputum and infected tissue for microscopy and culture. They are cultured on blood agar or Sabouraud agar for 3-14 days at 37°C in CO_2. The colonies are greyish white and dry. They are embedded

in the medium and difficult to remove. The casein hydrolysis test is used to differentiate *Nocardia* species. They are sensitive to sulphonamides, cotrimoxazole, rifampicin and dapsone.

GRAM NEGATIVE BACILLI

ENTEROBACTERIA

These Gram-negative rods belong to family enterobacteriaceae. Important genera are *Escherichia, Shigella, Edwardsiella, Salmonella, Arizona, Citrobacter, Klebsiella, Enterobacter, Hafnia, Serratia, Proteus, Providentia* and *Yersinia*.

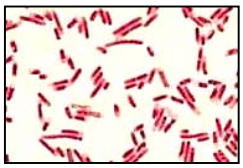
GENERAL CHARACTERS OF THE FAMILY

1. Gram negative rods
2. Non spore forming
3. If motile, they have peritrichous flagella
4. Facultative anaerobes
5. Catalase positive except S*higella dysenteriae* type I
6. Oxidase negative
7. Nitrate reducer
8. Ferment glucose with production of acid. Gas may or may not be produced
9. They can grow on MacConkey medium (bile salt containing media)
10. G+C DNA content is 39-59%
11. Antigens include:-
    a. Somatic or O cell wall antigens
    b. K or V capsular antigen
    c. H or Flagellar antigen

PATHOGENICITY

All enterobacteriaceae are potentially pathogenic for humans, as they release lipopolysaccarides after death that which can result in endotoxic shock. Patients with underlying disease, immunosuppression, mechanical or medical manipulation are susceptible.

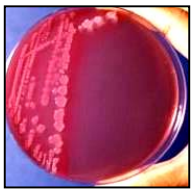
ESCHERICHIA COLI



These are the organisms normally found in the intestinal tract of humans and animals, but are also found in soil and water. They are Gram-negative motile rods (except a few strains) and are, non-spore forming.

CULTURAL CHARACTERISTICS

Escherichia coli is facultative anaerobe.



Optimum temperature for growth is between 35 to 37°C. On blood agar it yields 1-4 mm colonies that are round and have an entire edge. Some strains are haemolytic. On MacConkey agar the colonies are pink as they ferment lactose. Some strains are non-lactose fermenters and are non motile.

PATHOGENICITY

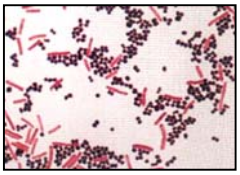
1. *E.coli* is a major cause of urinary tract infection and gastroenteritis.
2. Wound infections.
3. Meningitis, especially in infants.
4. Diarrhoea caused by following groups:
    a. Enteropathogenic *E.coli* (EPEC) (infantile diarrhoea)
    b. Enterotoxigenic *E.coli* (ETEC) (traveller's diarrhoea)
    c. Enteroinvasive *E.coli* (EIEC) (dysentery)
    d. Enterohaemorrhagic *E.coli* (EHEC, 0157: H7) (haemolytic uraemic syndrome in children)
    e. Diffuse adherent *E.coli* (DAEC) (diarrhoea)
    f. Enteroaggregative *E.coli* (EAggEC) (chronic diarrhoea)
    g. Diffuse adherent aggregative-adherent *E.coli* (DAAA) (diarrhoea)

These can be identified by agglutination reactions with commercially available antisera.

ANTIBIOTIC SENSITIVITY

The antibiotics used are ampicillin, fluoroquinolones, cotrimoxazole, nalidixic acid (in case of stool isolate), tetracycline, cephalosporins, aminoglycosides, aztreonam, pipracillin-tazobactam, imipenem and flouroquinolones.

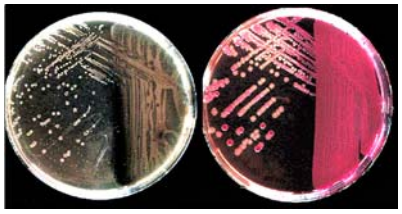
SHIGELLA

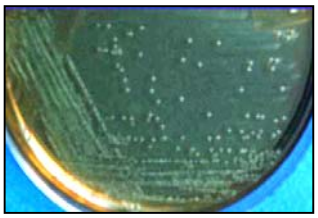


It is a Gram-negative, non-motile, enteric pathogen causing bacillary dysentery. It has four species; *Shigella dysenteriae, Shigella flexneri, Shigella boydii, Shigella sonnei*. These are further classified into serotypes. These are only found in intestinal tract of man. Chronic carriers are not known but after an attack of shigella dysentery, organism is excreted in stools for few weeks. Bacteria are acquired by ingesting contaminated food and water.

CULTURAL CHARACTERISTICS

They are non-motile, facultative anaerobes and catalase positive except *Shigella dysenteriae* type-I, which is catalase negative, does not produce H_2S. On blood agar, the colonies are 2-4 mm in size, entire and convex. On MacConkey agar, the colonies are pale, non-lactose fermenting. Other media used are deoxycholate citrate agar (DCA), Salmonella Shigella agar (SS agar) and xylose lysine deoxycholate agar (XLD agar). On all these media they produce non-lactose-fermenting colonies. Exception is *S.sonni*, which ferments lactose slowly. Strains or serotypes can be identified by agglutination reactions with commercially available anti-sera.





PATHOGENICITY

Shigellae are responsible for:

1. Bacillary dysentery
2. Meningism and other neurological symptoms. *Shigella dysenteriae* type-I produces a neurotoxin that enters blood and affects the central nervous system, causing meningism or even coma.

They differ from salmonella by remaining localised in the intestinal tract and cause intense inflammatory response. Differences between amoebic dysentery and shigella dysentery stools are shown in Table 17.2.

ANTIBIOTIC SENSITIVITY

The shigellae are susceptible to ampicillin, chloramphenicol, cotrimoxazole, tetracycline, nalidixic acid and fluoroquinolones (like ciprofloxacin and ofloxacin).

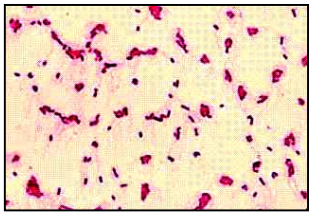
Table 17.2: Differences between Amoebic and Bacillary Dysentery

	AMOEBIC	BACILLARY
GROSS EXAMINATION		
Smell	Offensive	Odourless
Colour	Dark red	Bright red
Blood and Mucus	Mixed with faecal matter	Blood and mucus, no faecal matter
Reaction	Acidic	Alkaline
MICROSCOPIC EXAMINATION		
RBCs	Yellowish	Bright red
Pus cells	Scanty	Numerous
Macrophages	Few	Many with ingested RBCs
Charcot Leyden crystals	Present	Absent
Trophozoites	Entamoeba histolytica	No
Bacteria	Many motile	Not motile

SALMONELLA

Based on DNA analysis, there is only one species of the genus *Salmonella*, that is *Salmonella enterica*, which has seven subspecies. Most of the serotypes infecting mammals are in subspecies I. The various subspecies are:

I Enterica
II Salamae
IIIa. Arizonae
IIIb. Diarizonae
IV. Houtenae
V. Indica
VI. Bongori



The serogroups and serovar of subspecies enterica are shown in Table 17.4. There are more than 2200 serotypes of *Salmonella enterica*. They contain O or H antigens and most virulent strains contain a capsuler, the virulence or Vi antigen.

CULTURAL CHARACTERISTICS

They are facultative anaerobes. Optimum temperature is 35-37°C. They grow on ordinary media. Selenite-F and Tetrathionate broth are used as enrichment media inhibiting normal intestinal bacteria. After 24 hours, subcultures are made on differential and selective media like DCA, MacConkey and SS agar on which the colonies are pale and non-lactose fermenting. Bismuth sulphite, MacConkey agar is used for rapid detection. On this the colonies of *Salmonella typhi* are black because of H_2S production. On XLD salmonella forms pink colonies, while *S.typhimurium* and *S. paratyphi* C form red/ pink black centred colonies.

Table 17.3: Antigenic formulae of some common salmonellae according to Kaufman-White scheme.

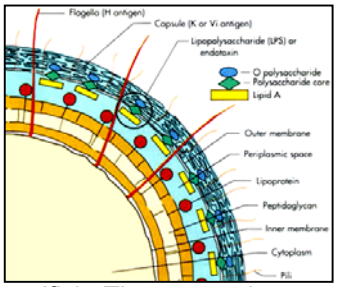
Serotype	'O' antigen	'H' antigen	
		Phase-1	Phase-2
Paratyphi A	1, 2, 12	a	[1, 5]
Paratyphi B	1, 4, [5], 12	b	1, 2
Typhimurium	1, 4, [5], 12	i	1, 2
Paratyphi C	6, 7, (Vi)	c	1, 5
Typhi	9, 12, (Vi)	d	-
Enteritidis	1, 9, 12	g, m	[1, 7]

Table 17.4: Serogroups and serovars of Salmonella enterica

SEROGROUP	SEROVAR
A	Paratyphi A
B	Paratyphi B, Typhimurium, Derby
C	Paratyphi C, Cholerasuis, Virchow
D	Typhi, Dublin, Enteritidis
E	Anatum

IDENTIFICATION

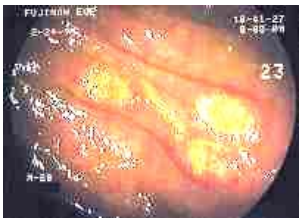
Antisera directed against the 'O' or somatic antigens and 'H' or flagellar antigens are used for slide agglutination tests. The suspension of the organisms from the culture is prepared in saline. A drop of this and a drop of antiserum is mixed on the slide and examined for agglutination, which should appear in 10-30 seconds. O antigens are cell wall or somatic antigens that identify groups of salmonellae from A to Z. H antigens are the antigens of the flagella and they are found in two phases, Phase-I (specific) and Phase-II (non-specific). These are the two antigenic forms of flagella. Some s*almonellae* can exist in both forms. Vi is heat labile, capsular antigen present only in capsulated organisms such as *S.paratyphi C, S.dublin* and *S.typhi.*



PATHOGENICITY

The salmonellae are capable of causing variety of conditions all referred to as salmonellosis. The three major categories of salmonellosis are

- Enteric (typhoid) fever
- Septicaemia
- Gastroenteritis



Typhoid Fever

S.typhi, S.paratyphi A, B, and *C* cause the disease. The infection occurs through oro-faecal route. Organisms pass the acidic barrier of stomach and enter the intestinal lumen. In the intestine, the organisms first attach to the epithelial cell and then through pinocytic movement enter the intracellular space. The organisms may multiply in the pinocytic vacuole and then pass to lamina propria from the other end of the epithelial cell. In the lamina propria the organisms enter the lacteals (small lymphatics) and through them to local lymph glands and thoracic duct. This opens into the blood stream and thus the organisms enter into blood stream. This is the stage of primary bacteraemia. The organisms are then carried to reticuloendothelial organs by the blood stream like liver, spleen, bone marrow, kidney, lymph nodes and Peyer's patches of the small intestine. The organisms multiply in these organs and after sufficient multiplication they enter the blood stream for the second time. This is the stage of secondary bacteraemia. This is the time when patient develops fever. The swelling of Payer's patches causes ulceration of the small intestine. Intestinal perforation sometimes occurs due to antigen-antibody hypersensitivity. The microorganisms multiply in intestinal lymphoid tissue and are excreted in stool. Blood culture is usually positive in the 1st week. Urine culture is positive in the 3rd and 4th week of illness. Stool culture is positive in 2nd to 4th weeks of illness.

Widal test: is the serological test to help in the diagnosis of the disease. It becomes positive after a week of illness. The titre rises after 7-10 days. Demonstration of riseing titre (4 fold) helps in diagnosis. Typhidot (an immunochromato-graphic technique) detects both IgG and IgM antibodies against a 60-kilodalton protein in the cell wall of *Salmonella typhi*. It is better than Widal but may show cross-reactions and false positivity, although it is sensitive but expensive.

Salmonella Enterocolitis

It is the most common form of salmonellosis which can be caused by any of the more than 2200 serotypes. Eating infected food usually causes it. The organisms are present in the gut of animals like hens and ducks. Infection can occur through infected meat or even infected eggs. After the ingestion of infected food, diarrhoea occurs consisting of 2-3 loose motions daily. The disease is usually self-limiting and does not require antibiotic treatment.

Salmonella carriers

It is the most common form of salmonellosis, caused by any of the more than 2200 serotypes. After enteric fever less than 2% of cases become chronic carriers (continue to excrete the organism in their faeces and urine even after one year). They harbour the organisms in their gall bladder and kidneys. These carriers serve as the source of infection for other individuals. Vi antibody titre is done to diagnose carrier state particularly of *S.typhi or S.paratyphi* C. A titre of 10 or more is considered significant.

ANTIBIOTIC SENSITIVITY

They are usually susceptible to fluoroquinolones (ciprofloxacin and ofloxacin etc.) and

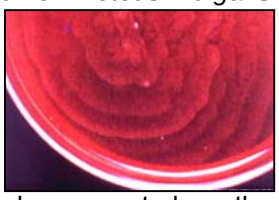
ceftriaxone. Many strains of *S.typhi* and *S.paratyphi A* have become resistant to chloramphenicol, ampicillin and cotrimoxazole and are known as MDR typhoid.

PROTEUS

The important species are *Proteus mirabilis* and *Proteus vulgaris*. They are normally found in the intestine of human beings and animals, in water and soil. These are Gram-negative rods, highly motile and non-spore forming.

CULTURAL CHARACTERISTICS

The media used for their growth are blood agar and MacConkey agar. They do not require enriched media for their growth. On blood agar, *Proteus mirabilis* and some *Proteus vulgaris* strains produce a **swarming growth** because of rapid motility. They spread on the surface of the medium forming a thin film. This **swarming** can be prevented on the medium by giving an alcohol wash or by increasing the content of agar in the medium. On MacConkey agar and medium deficient in salt (e.g., CLED) the colonies do not swarm. Proteus cultures give a specific fishy smell, non-lactose fermenting pale colonies, and rapid urea production.



PATHOGENICITY

These bacteria are seen as opportunistic pathogen and cause:

1. Urinary tract infections
2. Ear infections
3. Wound infections
4. Bacteraemia
5. Osteomyelitis

ANTIBIOTIC SENSITIVITY

Proteus is sensitive to gentamicin and other aminoglycosides and cephalosporins. Proteus is resistant to tetracyclines, sulphonamides and polymyxin. *Proteus mirabilis* may be sensitive to ampicillin while *Proteus vulgaris* is resistant. *P.vulgaris* is also resistant to first generation cephalosporins. Nitrofurantoin used for treating UTI is ineffective because of the alkaline *p*H of the urine in proteus infections.

PROVIDENCIA

They are motile, Gram-negative rods, which do not swarm. Three species are important, *Providencia rettgeri, Providencia stuartii*, *Providencia alcalifaciens.*

CULTURAL CHARACTERISTICS

They produce non lactose-fermenting colonies on MacConkey agar and need to be differentiated from shigella and salmonella. They are urease positive. They can grow on blood and nutrient agar.

PATHOGENICITY

They may cause infection of urinary tract, wounds and burns.

ANTIBIOTIC SENSITIVITY

They have susceptibility similar to proteus.

MORGANELLA

Morganella morgani is the only known species. It is motile, Gram-negative non-lactose fermenting, rod that does not produce swarming growth but is urease positive. It causes urinary tract and wound infections.

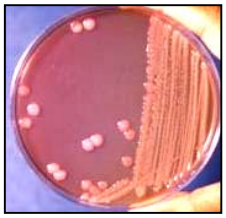
KLEBSIELLA

They are found in the intestinal tract of human beings and animals, soil and water. *Klebsiellae* are capsulated, non-spore forming, non-motile, Gram-negative rods and has following species and subspecies:

1. *Klebsiella ornithinolytica*
2. *Klebsiella oxytoca*
3. *Klebsiella planticola*
4. *Klebsiella pneumoniae*:
   a. Subsp.*pneumoniae*
   b. Subsp.*aerogenes*
   c. Subsp.*rhinoscleromatis*
   d. Subsp.*ozanae*

CULTURAL CHARACTERISTICS

They are facultative anaerobes and grow best at 37°C. On blood agar the colonies are 2-4 mm in size, high convex, mucoid and slimy. On MacConkey agar colonies are mucoid, slimy and lactose fermenting (pink). They are urease positive.



PATHOGENICITY

1. *Klebsiella pneumoniae*, sub-species *pneumoniae* causes pneumonia, nosocomial urinary tract infection, septicaemia, meningitis and wound infections.
2. *Klebsiella pneumoniae* subspecies *rhinoscleromatis* causes rhinoscleroma (chronic inflammatory growths of nose,

pharynx and upper respiratory tract) and causes deformity of the infected area.

3. *Klebsiella pneumoniae* subsp. *ozanae* causes atrophic rhinitis

ANTIBIOTIC SENSITIVITY

Antibiotics used are tetracyclines, cotrimoxazole, aminoglycosides, piperacillin, cephalosporins, fluoroquinolones, tazobactam and imipenem. *K.pneumoniae* is genetically resistant to ampicillin. Many strains produce Extended spectrum β-lactamase (ESBL) with makes them resistant to all β-lactams.

ENTEROBACTER

They are Gram-negative motile rods causing urinary tract and wound infection. They are susceptible to aminoglycosides, aztreonam, cotrimoxazole, third generation cephalosporins and imipenem, but can readily develop resistance due to ESBL production.

SERRATIA

They are Gram-negative, non-lactose fermenting rods and have all the characteristics of the family enterobacteriaceae. Few strains produce red-pigmented colonies. Species of medical importance are *Serratia marcescens*, *Serratia liquefaciens* and *Serratia rubidae*. These organisms are notorious for hospital-acquired (nosocomial) infections. They are usually resistant to many antibiotics and produce ESBL enzymes.

PSEUDOMONAS

They are Gram-negative rods, strict aerobes, motile with single polar flagellum except *Burkhulderia mallei*, which is non-motile. They are catalase and oxidase positive. Many are free-living species, and are plant pathogens. The species of importance are *Pseudomonas aeruginosa, Ps. putida, Ps. fluorescens* and *Ps. stutzeri.* Molecular analyses of the group have led to revised taxonomic classification. As a result, many species of the genus pseudomonas have been allocated new genera like *Burkholderia, Stenotrophomonas, Comamonas,* and *Brevundemonas e.g., Burkholderia (*previously *Pseudomonas) pseudomallei, Stenotrophomonas (*previously *Pseudomonas or Xanthomonas) maltophilia, Burkholderia* (previously *Pseudomonas) capacia.*

CULTURAL CHARACTERISTICS

They are strict aerobes. Optimum temperature for growth is 35-37°C but *Pseudomonas aeruginosa* can also grow at 42°C and on ordinary media, do not ferment glucose in O-F medium. Appear as pale non-lactose fermenting colonies on MacConkey's agar. They are common organisms found in hospital environment, water, soil and form part of flora of the intestine. *Pseudomonas aeruginosa* can grow in antiseptic solutions and in eye drops used in hospitals. The growth gives a sweet fruity odour. *Pseudomonas aeruginosa* produces two red, black, yellow or green, water-soluble pigments, fluorescence and pyocyanin diffusing into the medium. Colonies are usually flat with slightly irregular edges and the long axis of the colony is in line with the line of inoculation. Some strains produce haemolysis on blood agar. Cetrimide blood agar is a selective medium for *Pseudomonas aeruginosa.*

PATHOGENICITY

Pseudomonas aeruginosa produces several virulent factors including exotoxin A, proteases, leucocidin, and phospholipase C. It is a common organism of wound, ear and eyes infection. Later two may lead to meningitis. It causes urinary tract infection, pneumonia and septicaemia when introduced into the body by catheters. Healthy individuals are rarely infected. Patients having burns, cystic fibrosis, catheters and those on artificial ventilation in ICUs/CCUs are susceptible. *Burkhulderia mallei* cause glanders, a disease of horses accidentally transmitted to human beings. The infection starts as a skin ulcer, spreads through lymphatics to the blood stream. Recommended treatment is a combination of an aminoglycoside and tetracycline. *Burkholderia pseudomallei* cause melioidosis. It is an acute, or at times chronic lung disease, which may cause localised abscesses or bacteraemia. The disease is fatal if untreated. Chloramphenicol in combination with aminoglycosides or tetracycline is the drugs of choice.

ANTIBIOTIC SENSITIVITY

Pseudomonas by virtue of smaller pores in the cell wall are resistant to many antibiotics. Aminoglycosides, fluoroquinolones, ceftazidime, cefoperazone, piperacillin, ticarcillin, aztreonam and imipenem are used.

ALCALIGENES

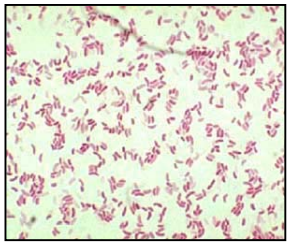
They are Gram-negative, motile aerobic, oxidase positive, rods, which produce alkalinity and therefore, dark green colour in the O-F medium. They act as opportunistic pathogens and can cause wound and urinary tract infections besides rare meningitis. They are sensitive to penicillins.

ACINETOBACTER

This organism belongs to the family neisseriaecae. These are Gram-negative, glucose non-fermenter, strict aerobe, non-motile, oxidase and nitrate negative cocco-bacilli or diplo-bacilli resembling neisseria in morphology. It is responsible for hospital-acquired (nosocomial) infections of wounds and urinary tract. Some species are highly resistant to most antibiotics and are very difficult to treat.

VIBRIO

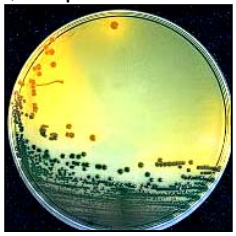
They are Gram-negative, oxidase positive, comma shaped organisms. The species of importance are *Vibrio cholerae* and *Vibrio parahaemolyticus*.



Vibrio cholerae are found in intestinal tract of carriers of cholera. Other vibrios are found in water, soil, seafood and sewage. They may appear as straight bacilli on certain solid media. They have a single polar flagellum and are highly motile (darting motility). They become non-motile if suspended in distilled water and hence motility should be tested in normal saline.

CULTURAL CHARACTERISTICS

They are facultative anaerobes and grow best at 35-37°C. They can grow best at a high *p*H (8.5-9.5) but acidic *p*H kills them. On MacConkey agar colonies are non-lactose fermenting. Alkaline peptone water is an enrichment medium used for initial culture and transport. Subcultures must be made within 6 hours, as proteus starts over-growing after this time. The colonies of *Vibrio cholerae* on selective medium (Thiosulphate Citrate Bile Salt Sucrose agar, TCBS) are yellow due to sucrose fermentation, which differentiate them from non-sucrose fermenting *V.parhaemolyticus*.



BIOCHEMICAL REACTIONS

Vibrio cholerae serotype O1 has two biotypes, Cclassical and *El tor* depending upon the biochemical reaction. The main differences between the *El tor* and classical vibrio cholerae are shown in Table 17.5. The classical and *El tor* biotypes share the same somatic antigens and hence are agglutinated with the same O1 antiserum. Both biotypes are further subdivided into three serotypes, Ogawa, Inaba and Hikojima. O139 is the other serogroup, which causes cholera like symptoms. Sometimes *Aeromonas hydrophila* causes confusion in diagnosis by showing similar morphology and biochemical reaction. O129 disk is used to differentiate. *Aeromonas hydrophila*, which is resistant to it whereas *Vibrio cholerae* are susceptible.

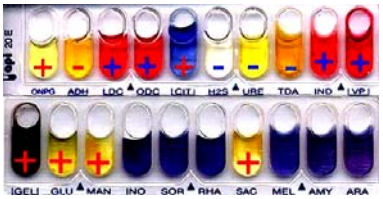


Table 17.5: Differences between Classical and El tor Vibrio cholerae.

CHARACTER	CLASSICAL	*El tor*
Chicken cell agglutination	-	+
Polymyxin sensitivity	S	R
VP test	-	+
Soluble haemolysin	-	+
Susceptibility to bacteriophage	+	-

PATHOGENICITY

The enterotoxin producing strains of vibrio cause cholera, which enter the intestinal epithelial cells and cause out pouring of fluid and electrolytes by stimulating adenyl cyclase and cAMP, leading to active secretion of chloride and bicarbonate ions alongwith massive quantities of water in the intestine leading to sever diarrhoea known as rice-water stools. Administration of intravenous fluids and electrolytes is critical for the recovery of the patient. The organisms enter the human body through oro-faecal route.

ANTIBIOTIC SENSITIVITY

Each biotype is sensitive to a wide range of antibiotics e.g., tetracyclines (particularly vibramycin), erythromycin, chloramphenicol, sulphonamides, nalidixic acid and fluoroquinolones.

V.parahaemolyticus is a murine vibrio, requires high salt concentration for growth. It causes acute enteritis associated with consumption of improperly cooked seafood.

CAMPYLOBACTER

Campylobacter species are curved, spiral, Gram-negative rods with a single polar flagellum. They grow best under microaerophilic conditions with 10% CO_2 on an enriched and special medium. *C.jejuni/coli* is an important human pathogen causing gastroenteritis, especially in children. The diarrhoea is usually self-limiting, but may last for several days.

ANTIBIOTIC SENSITIVITY

Macrolides (erythromycin, clarithomycine) and aminoglycosides are usually used.

HELICOBACTER

Helicobacter pylori is a spiral-shaped, Gram-negative rod, 0.5 x 3.0 µm in size. The catalase-positive organism has 4-6 sheathed flagella attached to one pole. *H.pylori* is a major cause of peptic ulcer disease and gastritis in humans. Bacteria most likely spread from person to person through the faecal-oral route or the oral-oral route, and humans are the primary reservoir for this infection. Several invasive and non-invasive tests are available to detect *H. pylori* in patients. These include specific *H. pylori* IgG antibodies, breath tests with ^{13}C or ^{14}C-labelled urea and endoscopy with biopsy.

ANTIBIOTIC SENSITIVITY

Therapy for *H. pylori* infection consists of 1-2 weeks of one or two effective antibiotics, such as amoxicillin, tetracycline, metronidazole, or clarithromycin, plus either ranitidine, bismuth citrate, bismuth subsalicylate, or a proton pump inhibitor.

AEROMONAS

They belong to the family vibrionacae. The important species is *Aeromonas hydrophila*. They are Gram-negative, motile, oxidase positive, non-lactose fermenting rods. They are normally found in water and soil. *A.hydrophila* can cause diarrhoea, meningitis and wound infections. They are sensitive to aminoglyco-sides, cephalosporins and tetracyclines.

PLESIOMONAS

They also belong to the family vibrionacae. They are Gram-negative, oxidase positive, motile, rods. The important species is *Plesiomonas shigelloides.* They can cause diarrhoea and wound infection. Table 17.6 shows the differences between the three genera of the family vibrionacea.

Table 17.6: Differences between genera of vibriacea

CHARACTERISTIC	VIBRIO	AEROMONAS	PLESIOMONAS
DNAse	+	+	-
Gas from glucose	-	+/-	-
Growth on TCBS	+	+/-	-
Inhibition by O/129	+	-	-

BACTEROIDES

They are Gram-negative, non-motile and non-spore forming, obligatory anaerobic, pleomorphic rods. Important species of this genus are *Bacteroides fragilis* and *Prevotella melaninogenicus*. They are normally found in the gastrointestinal tract of human beings. *Bacteroides fragilis* constitute bulk of faecal organisms by weight (99% of faecal flora), much more than *E. coli*. *Prevotella melaninogenicus* is found in GIT, mouth and vagina. They are usually long filamentous and form ciron bodies (dilated round structure).

CULTURAL CHARACTERISTICS

They are strict anaerobes and best isolated if the brain-heart infusion (BHI) agar medium containing 10% CO_2 has kanamycin, neomycin or gentamicin to which they are resistant. The growth usually takes 48-72 hours. *B.fragilis* gives pearl-grey or white colonies, which are smooth and glistening. Bacteroides are sensitive to metronidazole and resistant to gentamicin. These identification disks are placed on the primary culture plates. *Prevotella melaninogenicus* produces small, brown to black colonies, which give a pink-red fluorescence under UV light. Material on swabs containing the organism may show red fluorescence in UV light. *Bacteroides fragilis* does not produce any pigment. Colonies are small 1-2 mm and may show haemolysis.

PATHOGENICITY

B.fragilis is linked with infections that occur below the diaphragm. Infections are usually with mixed flora.

1. Wound infections
2. Deep-seated pus or abscess
3. Septicaemia
4. post-operative peritonitis
5. Gynaecological infections

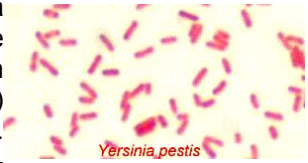
ANTIBIOTIC SENSITIVITY

They are sensitive to metronidazole, clindamycin and chloramphenicol *P.melaninogenicus* is sensitive to penicillins. All the anaerobes including *Bacteroides spp.* are resistant to gentamicin. *B.fragilis* produces β-lactamase and is resistant to penicillin and cephalosporins

except cefoxitin and cefotetan.

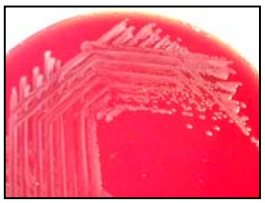
YERSINIA

They are pleomorphic Gram-negative rods or small cocco-bacilli, capsulated and show bipolar staining with Giemsa stain. All species are motile at room temperature (22-28°C) except *Yersinia pestis*. They become non-motile at 37°C. The species of importance are *Yersinia pestis, Yersinia enterocolitica* and *Yersinia pseudotuberculosis.* Rats are reservoirs for *Yersinia pestis*. *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are basically animal pathogens and the disease is transmitted to man through handling animals (zoonosis).



CULTURAL CHARACTERISTICS

They are aerobes and facultative anaerobes. They grow on ordinary media like blood agar and as non-lactose fermenters on MacConkey and Salmonella-Shigella agar. The colonies are usually greyish and mucoid. They are catalase positive and oxidase negative. They are very rapid urea splitters.



PATHOGENICITY

All Yersinia species possess O antigen, that is toxic for animals. *Y.pestis* has fraction I, V/W antigen and murine toxin. *Yersinia pestis* is a highly virulent causative organism of plague. It enters the body through the bite of infected rat flea. Human to human transmission may occur. From here it goes through the lymphatics to the regional lymph nodes where inflammation occurs so there is swelling of lymph nodes, usually of axillary region. These painful swollen nodes are called **Bubos (bubonic plague)**. From here the bacilli can invade the blood causing septicaemia and after that to various organs. Usually the lungs are infected and this form of disease is called **pneumonic plague**, an almost 100% fatal disease. In this the infected person is highly infective and infection spreads through droplets. *Yersinia pseudotuberculosis* causes enterocolitis and mesenteric lymphadenitis, which resembles acute appendicitis. *Yersinia enterocolitica* causes gastroenteritis, septicaemia severe arthritis 1-14 days after the acute attack. Some strains produce enterotoxin.

ANTIBIOTIC SENSITIVITY

Yersinia pestis is sensitive to tetracycline, chloramphenicol and fluoroquinolones. *Yersinia enterocolitica* is sensitive to sulphonamides, aminoglycosides and nalidixic acid and *Yersinia pseudotuberculosis* is sensitive to sulphonamides and penicillin.

PASTEURELLA

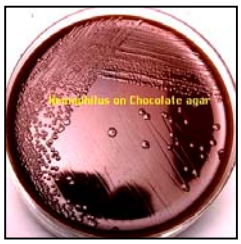
Pasteurellae are small, Gram-negative, cocco-bacillus, grown on ordinary media, forming small greyish colonies. These organisms are important members of indigenous flora of the respiratory tract or oropharynx of many animals and birds.It is part of the normal flora of mouth of cats and dogs. Humans are accidental hosts and man is infected by the bite of these animals. Important species is *P.multocida*. They are associated with severe, life-threatening systemic diseases involving both hemorrhagic pneumonia and septicaemia. The important species is *Pasteurella multocida.* It is sensitive to penicillin.

HAEMOPHILUS

The genus is characterised by an absolute requirement for blood components in its growth medium. They are small Gram-negative cocco-bacilli, long filamentous forms are also usually seen. They are non-motile. Some strains are capsulated. *Haemophilus influenzae* is the most important species because of its pathogenicity for humans causing respiratory and meningeal infections in children. *Haemophilus aegyptius*, *Haemophilus ducreyi* and *Haemophilus parainfluenzae* are others.

CULTURAL CHARACTERISTICS

It is a facultative anaerobe and grows best in CO_2 enriched environment at an optimum temperature of 35-37°C. They require two factors for their growth, X factor (haematin) and V factor (Nicotinamide Adenine Dinucleotide, NAD). These factors are present in blood, hence best medium for their growth is blood agar and chocolate agar is even better. The greyish-white, mucoid colonies are small, 0.5 mm in size. *Staph aureus* produces V factor and hence the growth of *Haemophilus* around the staphylococcus colonies is increased. This phenomenon is called **satellitism**. *Haemophilus influenzae* requires both X and V factors for its growth while *H. parainfluenzae* only requires V



factor, and *H. ducreyi* only requires X factor.

PATHOGENICITY

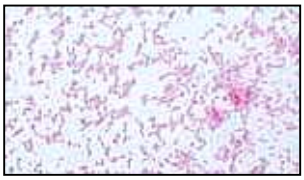
Haemophilus influenzae is divided into six serotypes, e.g., a-f. Type b is the most common cause of infections in children. Non-capsulated strains are usually virulent. *H.influenzae* causes pyogenic meningitis in children, bacteraemia, acute epiglottitis, middle ear infection and pneumonia. *H.aegyptius* causes epidemic form of conjunctivitis (pink eye). *H.ducreyi* causes genital sore (chancroid or soft chancre), which is transmitted sexually.

ANTIBIOTIC SENSITIVITY

Most of the *Haemophilus* species are ampicillin sensitive. The strains producing β-lactamase are emerging, the next drug of choice is chloramphenicol/ceftriaxone especially in meningitis. Vaccine containing capsular polysaccharides (Hib) is protective. Erythromycin, cephalosporins and fluoroquinolones are also effective.

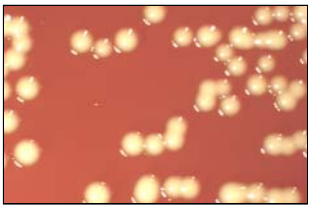
BRUCELLA

They are small, Gram-negative, rods or cocco-bacilli. Strains of medical importance are *B.abortus, B.melitensis* and *B.suis.* They are basically animal pathogens. Brucella species are the etiological agents of brucellosis in livestock *B.melitensis* is a pathogen of goats and sheep, *B.abortus* of cattle and *B.suis* of pigs.



CULTURAL CHARACTERISTICS

They are aerobic and *Brucella abortus* requires 5-10% CO_2 enriched environment for growth. Optimum temperature is 35-37°C. The organisms are difficult to isolate from blood culture that requires incubation for 4-6 weeks. Media used for isolation are brucella agar and trypticase soya agar. The colonies on solid media are small, convex, smooth, 1-2 mm, appear after 2-3 days of subculture.



PATHOGENICITY

Brucellae have two major surface antigens, A and M. Human infections usually occur through animal source and are common in persons who deal with animals or are in contact with them (occupational hazard). The common routes of infection are intestinal tract (ingestion of infected milk), mucous membranes (droplets) and skin (contact with infected tissue of animal). The disease produced is called brucellosis, undulant fever or Malta fever. It is a chronic disease characterised by fever, weakness, myalgia and body pains especially backache and arthritis. The organisms are intracellular in reticuloendothelial system. Liver, spleen, lymph nodes and bone marrow are infected. The incubation period is 1-6 weeks. Relapses are common. In brucellosis the antibodies start to appear in serum after 7-10 days of fever and measurement of these antibodies helps in the diagnosis of brucellosis. These can be measured by slide agglutination test, tube agglutination test, complement fixation tests, Coomb's test and mercaptoethanol test.

ANTIBIOTIC SENSITIVITY

The organisms are susceptible to streptomycin, fluoroquinolones and tetracyclines. They are given in combination with rifampicin, which is the drug of choice.

BORDETELLA

They are Gram-negative cocco-bacilli, stain poorly with Gram's method. Important species are *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchoseptica*.

CULTURAL CHARACTERISTICS

Bordetellae are strict aerobes. Special media are required for their growth. These are Bordet-Gengou Penicillin medium, Charcoal cephalexin blood agar (CCBA) and blood agar. Best is CCBA medium. 5-10% CO_2 enhances the growth. The growth usually appears in 3-6 days. Colonies are 1-3 mm in size greyish, glistening pearl like and mucoid. They resemble mercury drops.

PATHOGENICITY

B.pertussis is an obligate human parasite, produces several virulence factors including histamine sensitising factor and pertussis toxin, which causes whooping cough syndrome.

Table 17.7: Biochemical reactions of Bordetellae

CHARACTER	*B.pertussis*	*B.para-pertussis*	*B.broncho-septica*
Motility	-	-	+
Oxidase	+	-	+
Catalase	+	+	+
Growth on Blood agar	_	+	+

LABORATORY DIAGNOSIS

The organism is difficult to isolate. Cough plate and postnasal swabs are unsatisfactory because of overgrowth of commensals. Per nasal calcium alginate cotton swab must be used as cotton inhibits growth. Culture plate should be inoculated immediately. The use of transport medium reduces the isolation rate.

SEROLOGY

It is helpful in diagnosis. Antigen is detected by immunofluorescence and antibody is detected by ELISA method. PCR is also being employed for the rapid diagnosis.

BIOCHEMICAL REACTIONS

Biochemical reactions of Bordetella are shown in Table 17.7.

PATHOGENICITY

It causes pertussis or whooping cough.

ANTIBIOTIC SENSITIVITY

Bordetellae are sensitive to erythromycin, tetracycline, chloramphenicol and cotrimoxazole, but is usually given to prevent secondary infection.

PROPHYLAXIS

Pertussis vaccine is given along with diphtheria and tetanus vaccine to children (DPT) in the normal vaccination programme of EPI. Immunity acquired by vaccination is not permanent.

Table 17.8: Biochemical reactions of enterobacteriaceae and other gram-negative bacilli[1]

	Lact	Suc	Glu	Man	Cit	MR	VP	Ind	Urea	Phenyl	H_2S	Mot	Ox	Cat	Gas
Esch coli	+	d	+	+	-	+	-	+	-	-	-	+	-	+	+
Klebsiella pneumoniae	+	+	+	+	+	-	+	-	+	-	-	-	-	+	+
Enterobacter spp.	+	+	+	d	+	-	+	-	-	-	-	+	-	+	+
Citrobacter freundii	+	d	+	+	+	+	-	-	d	-	d	+	-	+	+
Serratia spp.	d	+	+	+	+	+	d	-	d	-	-	+	-	+	+
Proteus vulgaris	-	+	+	-	+	-	-	+	+	+	+	+	-	+	+
Proteus mirabilis	-	d	+	-	+	-	-	-	+	+	+	+	-	+	+
Morganella morgani	-	-	+	-	-	-	-	+	+	-	+	d	-	?	d
Providencia sp	-	d	+	d	+	-	-	+	d	+	-	+	-	+	d
Salmonella typhi	-	-	+	+	-	+	-	-	-	-	+	+	-	+	-
Salmonella paratyphi A	-	-	+	+	-	+	-	-	-	-	-	+	-	+	+
Other Salmonella spp	-	-	+	+	d	+	-	-	-	-	d	+	-	+	d
Shigella spp	-	-	+	+	-	+	-	+	-	-	-	-	-	+	d
Y.enterocolitica	-	+	+	+	-	-	-	d	+ slow	-	-	+	-	+	
Vibrio cholerae	-	+	+	+	d	-	d	+	-	-	-	+	+	+	
V.parahaemolyticus	-	-	+	+	d	-	d	+	-	-	-	+	+	+	
Ps. Aeruginosa	-	-	+	-	+	-	-	-	-	-	+	+	+	+	

1 KEY: '+' = Positive reaction, '-' = Negative reaction, 'd' = Variable reaction, 'Lact' = Lactose fermentation, 'Suc' = Sucrose fermentation, 'Glu' = Glucose fermentation, 'Man' = Mannitol fermentation, 'Cit'.= Citrate utilisation, 'MR' = Methyl Red reaction, 'VP' = Voges Proskauer reaction, 'Ind' = Indole production, Urea = Urease production, 'Phenyl' = Phenylalanine decarboxylation, 'H2S' = H2S production, 'Mot' = Motility, 'Ox' = Oxidase production, 'Cat' = Catalase production

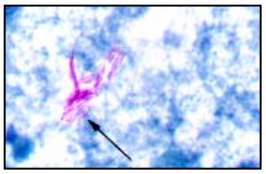
18. MYCOBACTERIA

Mycobacteria are aerobic, rod-shaped bacteria containing large amounts of complex lipids in their cell walls. These lipids make staining difficult and interfere with subsequent decolourisation in Z-N staining because they resist acid-alcohol wash and are therefore, referred as **acid-fast**. This genus is responsible for many important human diseases. The species of medical importance are grouped into:

1. The Mycobacterium tuberculosis complex
   a. *Mycobacterium tuberculosis*
   b. Mycobacterium bovis (including BCG)
   c. *Mycobacterium microti*
   d. *Mycobacterium africanum*
2. Atypical mycobacteria
   a. *Mycobacterium kansasii*
   b. *Mycobacterium intracellulare*
   c. *Mycobacterium avium*
   d. *Mycobacterium fortuitum*
   e. *Mycobacterium marinum*
   f. *Mycobacterium chelonei*
   g. *Mycobacterium malmoense*
   h. *Mycobacterium simiae*
3. Non- cultivable mycobacteria
   a. *Mycobacterium leprae*

MYCOBACTERIUM TUBERCULOSIS AND ATYPICAL MYCOBACTERIA

They are rod-shaped organisms and stained with Ziehl-Neelsen method for acid-fast bacilli. The property of acid fastness is due to waxes and fatty acids (mycolic acid) in their cell wall. They stain with difficulty with Gram stain and if stained, are weakly Gram-positive. *Mycobacterium tuberculosis* is acid fast with 20% sulphuric acid.



CULTURAL CHARACTERISTICS

Mycobacteria are difficult and slow to grow and time taken for their growth in artificial media is longer than any other bacteria because of the long doubling (generation) time (18 hours). Mycobacteria require protein rich media specially proteins of egg or serum. They are aerobic organisms. The optimum growth requirements of different mycobacteria differ and are accordingly divided into three classes:

1. Thermophilic which grow best at 44°C *(M.xenopi* and *M.avium-intracellulare*)
2. Mesophilic that grows best at 32-37°C *(M.tuberculosis* and *M.bovis).*
3. Psychotropic that grows best at 25°C (*M.chelonei, M.ulcerans* and *M.marinum*).

All are slow growers (require 4-8 weeks) except *M. fortuitum* and *M.chelonei*, which are rapid growers (<1 week i.e., 3-6 days.). On the pabsis of **Runyon** classification i.e., according to the production of pigment in relation to light and darkness mycobacteria are divided into:

1. Scotochromogens, which produce pigment whether in light or in dark *(M.scrofulaceum, M.szulgai).*
2. Photochromogens, which produce pigment only when exposed to light (All except those in other two groups i.e. *M.kansassi, M. marinum, M.simiae).*
3. Non-chromogens that do not produce pigment whether in light or dark (*M. avium-intercellulare*).

To check whether they produce pigment on exposure to light or not, the growth is exposed to light for 1-2 hours (not to direct sunlight) and re-incubated. The pigment of yellow or yellow orange colour will appear in next 18-24 hours.

Lowenstein Jensen medium, Dorset's egg medium, Middle Brook medium and Kirchner's media are used for growing mycobacteria. Lowenstein Jensen medium with pyruvate and glycerol is commonly used. The growth obtained is raised, dry, wrinkled, white or cream coloured, and if pigment appears it is of yellow to orange colour. The specimens are homogenised and decontaminated prior to inoculation using NaOH (Petroff's method). Various modifications of the procedure are employed. The culture bottles are examined weekly for growth. A positive culture takes about 4-8 weeks. Automated systems have been introduced to decrease the time of isolation of *M. tuberculosis* (see BACTEC RADIOMETRIC SYSTEM on page 59, and BACT ALERT on page 59).

DIAGNOSTIC TECHNIQUES

Following techniques are available for diagnosis of *M. tuberculosis* in clinical specimens:

1. Direct tests

a. Ziehl-Neelsen Staining

b. Auramine-phenol fluorescent staining which is more sensitive than Z-N staining.
c. DNA Hybridisation (PCR)
d. Cell wall Lipids determination by Gas liquid chromatography
e. Cell wall Antigen (tuberculostearic acid) in sputum
f. Culture
   i) Conventional and special techniques (Bactec, MGIT or Mico.MGIT))
   ii) Microagar technique
   iii) Microbroth technique
   iv) Guinea pig inoculation

2. Indirect tests

a. Histopathology of different tissues including FNAC of lymph nodes
b. Serum protein electrophoresis
c. Radioactive bromide shift (partition) test (CSF) (ratio of serum and CSF bromide <1.6 to 1)
d. Tuberculin skin testing
e. Serological diagnosis: MycoDot™, serological assay detects anti-mycobacterial antibodies in serum for active tuberculosis. The test can be performed on venous or capillary blood, plasma, or serum. It takes only 20 minutes to perform and requires no special equipment. Results of the MycoDot serological assay can diagnose suspected cases of pulmonary and extrapulmonary tuberculosis.
f. Mycobacteriophage assay (Fast-plaque) This is a new technique. The bacteriophages are mixed with sputum specimen; the mixture is dealt with anti bacteriophage, which will destroy the phages not taken up by the mycobacteria. The mycobacteria if present are then lysed. Rapid growing mycobacteria are then used to take up released phages and are allowed to grow on agar plate. If there is plaques formation then it is assumed that initial specimen had mycobacteria. The test result is usually available within 2 days.

Enumeration of AFB on Ziehl-Neelsen (Z-N) stained smears

Number of bacteria present in the smear can be described quantitatively as well as the percentage of live bacteria. The latter will help to determine the therapeutic response in subsequent smear examination.

Table 18.1: Bacteriological index

No of Organism	Significance
1-2 per entire smear	Doubtful (repeat)
3-9 per entire smear	Rare (1+)
≥10 per entire smear	Few (2+)
≥ 1 per oil immersion field	Numerous (3+)

Morphological index

It is percentage of live mycobacteria in a smear. Usually 200 free, pink, mycobacteria are counted and number of live bacteria is determined (Table 18.1).

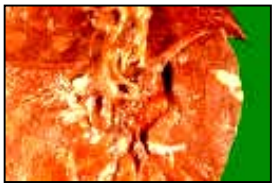
IDENTIFICATION

The organisms can be identified by their rate of growth, pigment production and the growth pattern. Following tests will help in identification of species:

1. Growth on PNB (paranitrobenzoic acid)
2. Growth on TCH (thiophen-2-carboxylic acid hydrazide)
3. Growth on Sauton agar
4. Niacin test (*M.tuberculosis* is positive)
5. Urease test (*M.tuberculosis* is positive)
6. Catalase test
   a. Catalase test at 68°C (*M.marinum* is positive)
   b. Semiquantitative Catalase (>45 mm *M kansasii* is positive)
7. Nitrate reduction (*M.tuberculosis* is positive)
8. Growth rate
9. Pigment production
10. Growth at 25°C, 30°C, 40°C and 45°C
11. Arylsulphatase activity (*M.fortuitum* is positive)
12. Tween 80 hydrolysis (*M.kansasii* is positive)
13. Tellurite reduction test (*M.avium* is positive)
14. Phage typing: Type A, B, C or BCG is resistant to phage 33D.

PATHOGENESIS

Mycobacterium tuberculosis and *M.bovis* are pathogenic for human beings and *M.bovis* for animals. The main source of infection is the infected person (usually through respiratory tract by small droplet nuclei) and the cattle (through infected un-pasteurised milk). Tuberculosis is of two types; primary and secondary. Primary tuberculosis occurs when a person is exposed to the tubercle bacilli and the organism multiply in the lungs and there is enlargement of the draining lymph nodes. This is called **Gohn's complex or primary complex** and usually occurs in childhood. Secondary tuberculosis is the one in which the



person who had primary infection is re-exposed to tubercle bacilli or there is reactivation of the primary lesion. Tuberculosis can affect any organ or tissue and may even be generalised called **miliary tuberculosis**. The main lesion is **granuloma** that may caseate, rupture and heal by fibrosis. Caseation and rupture of neck glands is commonly seen. *M.ulcerans* and *M.marinum* cause chronic nodular skin lesions and ulcers (swimming pool granulomas) usually from contaminated water. *M.kansasi* causes pulmonary infection similar to tuberculosis. *M.avium* and *M.intracellulare* usually cause pulmonary disease in AIDS patients. *M.scrofulaceum* causes cervical lymphadenitis in children. *M.fortuitum-M.chelonae* is saprophytic organisms that may cause chronic progressive pulmonary infection in patients with underlying lung disease. Many also cause injection abscesses and nosocomial infections.

TUBERCULIN SKIN TEST

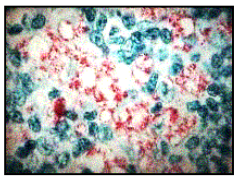
Purified protein derivative of *Mycobacterium tuberculosis* (PPD) is used to detect hypersensitivity of the individual to tubercle bacilli. (see MANTOUX TEST on page 230).

ANTIBIOTIC SENSITIVITY

First line or primary drugs are streptomycin, isoniazid, paraaminosalicylic acid, ethambutol, pyrazinamide and rifampicin. Treatment is a combination of 2 or more drugs for long duration (6 months to 2 year, depending upon site and severity of infection). Atypical mycobacteria are resistant to most of these drugs. Multi drug resistant strains of *M. tuberculosis* are emerging and newer drugs like ofloxacin, ciprofloxacin, cycloserine amikacin and kanamycin are being used.

MYCOBACTERIUM LEPRAE

They are absolute pathogens and are curved, rod-shaped acid-fast organisms like *Mycobacterium tuberculosis* but are less acid fast i.e., they are treated with 5% acid and are decolourised if 25% acid is used. They do not grow on artificial media but can grow in animals like the footpad of mice and armadillos. Demonstrating AFB in nasal and slit-skin smears usually confirms the diagnosis.



PATHOGENESIS

The epidemiology of leprosy is not well understood. Disease probably is contracted as a result of prolonged contact with infected individuals, who shed large number of organisms from infected lesions. Route of infection is the nose and upper respiratory tract or organisms enter through the skin. Sources of infection are nasal and respiratory secretion of the infected persons. Leprosy does not spread by short-term contact, its transmission is slow and a long time is required. Leprosy is a chronic disease involving nerves and skin. It is of two types, lepromatous and tuberculoid. The main difference is in the immune response. In **tuberculoid type**, there is a good immune response and the lepra bacilli are not found in the lesions, which are raised skin lesions with palpable thickening of peripheral nerves and focal area of anaesthesia. In **lepromatous** leprosy the immune response of the person is inadequate, hence there are many lepra bacilli in the lesions and nasal secretion. There is extensive skin involvement of ear lobes, forehead and nose (**leonine facies**). Due to the nerve involvement the patient cannot feel pressure and pain. Intermediate (borderline) types also occur.

Processing of smears

The laboratory usually receives, slit-skin and nose-blow smears and nasal scrapings for demonstration of the organisms. Modified Ziehl-Neelsen staining is used in that the decolourisation is done by 5% sulphuric acid or 3% acid (hydrochloric acid) alcohol.

Bacteriological Index

The bacteriological index indicates the density of leprosy bacilli in the smears and includes both living (solid staining) and dead (fragmented or granular) bacilli. Using oil immersion objective following scale is recommended for reporting:

Index	No of bacilli in oil immersion fields
0	None in 100
1+	1-10 in 100
2+	1-10 in 10
3+	1-10 in each
4+	10-100 in each
5+	100-1000 in each
6+	>1000 bacilli (many globi) in each

Morphological index

The morphological index is the percentage of presumably living bacilli to the total number of bacilli in the smear. It is usually calculated after examining 200 pink stained free (i.e. not in clumps) standing bacilli.

Antibiotic treatment

Standard drugs for treatment of leprosy are dapsone, rifampicin and clofazimine.

19. SPIROCHAETES AND SEROLOGY OF SYPHILIS

Spirochaetes are slender, spiral shaped organisms having cytoplasm, cell wall and outer membrane and between them there are axial filaments, which pull the organism into spiral form. These are also important for motility of the organism. *Treponema*, *Leptospira, and Borrelia* are included in this family.

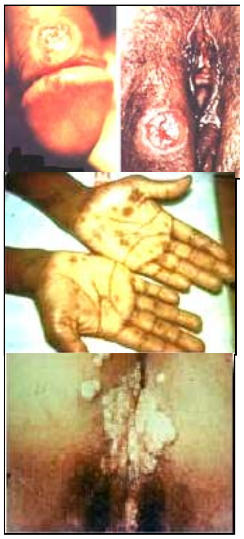
TREPONEMA

The treponeme of medical importance is *Treponema pallidum* that causes syphilis. This organism is not easily stained and hence is seen under dark ground illumination or with fluorescent labelled antibody or sliver stain. It is actively motile showing rotating movements. The coils in the spiral are evenly spaced. The organism has not been cultured in artificial media but can be grown in rabbit's testes. It also remains viable for 24 hours in blood stored at 4°C. Hence, serological tests are more important for the diagnosis of syphilis along with the demonstration of organisms in a clinical specimen by dark ground illumination.

PATHOGENICITY

It is either congenital syphilis (the baby is infected *in utero* because of the infected mother) or acquired syphilis. The latter is a sexually transmitted (venereal) disease (STD) and has three stages:

Primary syphilis: The chancre or ulcer appears on the external genitalia of male or female after 1-2 wks after initial contact. It is not painful and heals spontaneously.



Secondary syphilis: It occurs 6-8 weeks after the primary infection. The organisms enter the blood stream, cause a skin rash, and mouth ulcers.

Tertiary syphilis: In this stage the granulomas known as gumma appear in various organs. If nervous system is involved, it is called neuro-syphilis and causes general paralysis of insane or tabes dorsalis. Cardiovascular lesions causing aortic aneurysms are also common.

Congenital syphilis: Often the foetus dies and pregnancy is aborted. Maculopapular rash and jaundice appear in survivors. In untreated cases it leads to blindness, deafness and severe brain damage.

LABORATORY DIAGNOSIS

Active primary and secondary stage

Direct observation of *T.pallidum* by dark field microscopy or indirect immunofluorescence is possible.

Other stages

Diagnosis is dependent upon serological techniques.

SEROLOGY OF SYPHILIS

Three types of antibodies appear in the serum of the patient of syphilis. There are antibodies produced against non-treponemal antigens, treponema genus specific and species-specific antigens.

Antibodies against non-treponemal antigens

The antibodies are produced due to tissue damage and are called cardiolipin antibodies. These antibodies are non-specific and can appear in many other infections. These are assayed to monitor the response of the disease to therapy, as their titre tends to fall when treatment stops tissue damage. The antigen used in these tests is cardiolipin. Various tests based on these antibodies are:

- Wasserman and Kahn test (obsolete)
- VDRL test (slide flocculation)
- RPR (Rapid Plasma Reagin) test, (Agglutination test)

Antibodies against treponemal genus specific antigens

These antibodies reflect the presence of any of the Treponema antigen that may also be other than *T.pallidum*. Test based on these antibodies is Reiter Protein Complement Fixation (RPCF) test. The Reiter strain is an avirulent strain of *T.pallidum*. This test may be positive in bejel, yawn and pinta.

Table 19.1: Interpretation of tests in Syphilis

STAGE OF DISEASE	NON-TREPONEMAL ANTIGEN TESTS (VDRL)	TREPONEMAL ANTIGEN TESTS (TPHA)
Early	+ or -	+ or -
Secondary	+ or -	+
Treated	- or falling titre	+, Takes many years to become negative.

Antibodies against treponemal species specific antigens

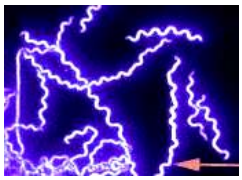
These antibodies are species specific and are directed against the antigens of *Treponema pallidum*. Tests based on these are:

1. TPHA (*Treponema pallidum* haemagglutination test)
2. FTA-ABS (Fluorescent treponemal antibody absorption test) Nictiol's strain is used in this test.
3. TPI (*Treponema pallidum* immobilisation test). This is costly or cumbersome to perform in routine laboratories.
4. 19s-IgM-FTA-ABS test
5. Various types of enzyme immunoassays
6. PCR is being employed in diagnosis especially of neurosyphilis.

ANTIBIOTIC SENSITIVITY

The organisms are sensitive to penicillin and it is the drug of choice in the treatment of syphilis.

BORRELIA

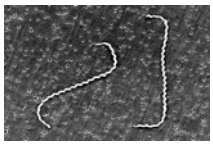


These are larger than treponemes and have irregular coils. Motility is both, by a rotating and a twisting motion. They are weakly Gram-negative. They stain with aniline dyes and can be observed with light microscope. They are difficult to grow in artificial culture media. *B.recurrentis* can survive for several months at 4°C. They can be passed transovarially in ticks and survive long period of time in these arthropods. They are sensitive to penicillin and tetracycline. The important organisms are:

1. *Borrelia recurrentis* causes louse borne relapsing fever.
2. *Borrelia vincenti* causes Vincent angina.
3. *Borrelia duttoni* causes tick borne relapsing fever.
4. *Borrelia burgdorferi* causes Lyme disease.

LEPTOSPIRA

Leptospira spp is characterised by appearance of a thin, very tight coiled spiral with a hook on one or both ends. They move actively and are difficult to stain, hence are seen by dark ground or phase contrast microscopy. Examination for leptospira in specimen like urine and CSF requires special methods.



CULTURAL CHARACTERISTICS

Leptospira spp are obligate anaerobes. The organisms are difficult to culture. The medium used is semisolid Tween albumin Fletcher's medium supplemented with bovine or rabbit serum. The optimum growth temp is 28-30°C. The cultures are examined weekly by dark ground microscopy.

PATHOGENICITY

They cause leptospirosis. The main species causing leptospirosis is *Leptospira interrogans.* It has many medically important serotypes, e.g., *Leptospira icterohaemorrhagica* associated with rats. These organisms usually infect both, wild and domestic animals. In human beings the disease presents like a viral illness with high fever, body aches and pains, jaundice or meningitis. If there is jaundice and renal failure, the disease is called Weil's disease. The diagnosis of leptospirosis is usually made serologically. The antibodies appear after the first week of infection. Urine and CSF should also be examined for the organisms. Urine is collected in buffered saline with *p*H 7.2 and is examined within one hour. The urine testing has to be repeated because the leptospira are passed intermittently in small numbers. The urine is centrifuged at slow speed for 5 min to remove the urinary cells, casts etc. The supernatant is again centrifuged at high speed for 15 min to concentrate the organism. The sediment is taken and examined by dark ground microscopy.

ANTIBIOTIC SENSITIVITY

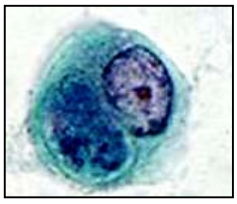
Penicillin is the drug of choice. Streptomycin, erythromycin and tetracycline can also be used.

20. CHLAMYDIA, RICKETTSIA, MYCOPLASMA

CHLAMYDIA

The genus chlamydia consists of obligate intracellular parasites, once believed to be large viruses. These bacteria have a cell wall resembling Gram-negative bacteria, but differ in certain properties. Like bacteria they replicate by binary fissure and contain both DNA and RNA, but lack peptidoglycan in their cell wall and ability to form ATP. They are sensitive to antibiotics. Chlamydiae of medical importance are *Chlamydia trachomatis, Chlamydia psittaci,* and *Chlamydia pneumoniae.* (The old name was TWAR strain from Taiwan (TW) and acute respiratory (AR).

MORPHOLOGICAL CHARACTERISTICS



Chlamydiae are small, round to oval, intracellular organisms that show morphological variation during their replication cycles. They reproduce in host cells and are visible as blue-mauve or mauve inclusion bodies. They are stained with Giemsa stain. In Gram smear they stain very weakly Gram-negative. They are seen by immunofluorescence technique in conjunctival scrapings.

CULTURAL CHARACTERISTICS

The chlamydia cannot be grown on artificial medium. They grow in the yolk sac of 6-8 day's chick embryo, which dies 4 days after inoculation. For isolation of Chlamydiae from clinical specimens, cell culture is used (McCoy cells lines). Cells are observed for intracellular inclusion bodies.

PATHOGENICITY

Chlamydiae primarily infect epithelial cells of mucous membranes or lungs. *Chlamydia trachomatis* (types A, B, C), biovar trachoma causes trachoma involving conjunctivae and corneae and can lead to blindness due to corneal opacities. Genital infection by chlamydiae (D-K serovars), biovar occulogenital causes urethritis in men, pelvic inflammatory disease and infertility in women and conjunctivitis in both sexes. Other serotypes (L1–L3) of *Chlamydia trachomatis*, biovar lymphogranuloma venereum causes sexually transmitted infections. The infection starts as a genital ulcer and causes lymphogranuloma venereum (LGV). C*hlamydia psittaci* is originally an animal pathogen. Inhalation of the organisms from faeces of animals or birds causes pneumonia in humans. *Chlamydia pneumoniae* (TWAR strain) causes atypical pneumoniae.

SEROLOGY

The genus-specific, species-specific or serotype-specific antibodies are helpful in the diagnosis. Complement fixation test or immunofluorescence technique is used.

DIAGNOSIS

Direct antigen identification by methods e.g., immunofluorescence and ELISA are rapid and reliable. In addition to serology, cell culture techniques may be used for the diagnosis. PCR is also employed for diagnosis.

ANTIBIOTIC SENSITIVITY

They are sensitive to tetracyclines, erythromycin, rifampicin, quinolones and chloramphenicol.

RICKETTSIA

They are bacteria but unlike bacteria, they are obligate intracellular parasites. They can only survive inside living cells. They should not be classified with viruses because they have all the properties of bacteria. Rodents and rats are the animal reservoir of Rickettsiae. Man is infected through bite of infected louse, flea or ticks (which have been feeding on these animals). Pathogenic Rickettsia are divided into the spotted fever group and the typhus group. Rickettsiae of medical importance are detailed in Table 20.1.

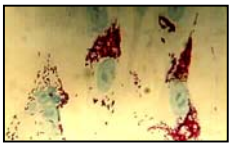
Table 20.1: Rickettsia of medical importance.

ORGANISM	DISEASE	HOST	VECTOR
R.prowazeki	Epidemic typhus	Man	Body louse
R.typhi	Murine typhus	Rat	Rat flea
R.tsutsugamushi	Scrub typhus	Rodents	Mite
R.rickettsi	Spotted fever	Dog	Tick
Coxiella burnetti	Q fever	Cow, goat and rodents	Aerosols and milk

MORPHOLOGICAL CHARACTERISTICS

They do not readily stain with Gram stain, but

can be visualised readily in tissues with Giemsa stain, seen as intracellular cocco-bacilli or rods.



PATHOGENICITY

The rickettsiae cause typhus, the type depending upon the organisms transmitted by louse, mites, or ticks. The patient develops high-grade fever, rash and body aches. The organisms multiply in the blood vessels. The untreated infection can lead to gangrene of fingers, brain damage and death. A recrudescence or re-infection of louse-borne fever later in life is known as Brill-Zinsser disease. The attack is milder than the previous illness.

CULTURAL CHARACTERISTICS

They are grown in embryonated hen eggs.

SEROLOGY

Serology of Rickettsial diseases is important, as the organisms are difficult to grow. Following tests are done:

1. **Weil-Felix reaction:** Antibodies against rickettsiae react with antigens of Proteus OX2, OX19 and OXK in agglutination test. Diagnostic findings with these antigens are shown in Table 20.2 Weil-Felix is non-specific test having false negative and false positive reactions. A rising or a single high antibody titre is presumptive evidence of infection.
2. **Complement fixation test:** The complement fixing antibodies (phase I and II) detected by microagglutination technique is useful for identification of Q-fever (*Coxiella burnetii*). A rise in complement fixing antibodies titres between acute and convalescent sera is diagnostic.
3. **Immunofluorescence test**: It is the most useful test for serological diagnosis of rickettsiae as it detects specific antibodies.
4. **Animal Pathogenicity**: Adult male guinea pig is given intraperitoneal injection of 2-4 ml blood from febrile patient. The response of guinea pig to rickettsial infection is fever (rectal temperature ≥40°C). *R.typh*i and spotted fever group produce an intense inflammation of the testes and scrotum, not seen in *R.prowazekii* or *Coxiella burnetii*. White mouse is used for *R.tsutsugamushi* infection. Rickettsiae may be demonstrated by Giemsa stain or by immunofluorescence in impression smears from tunica, spleen or liver of these animals.

ANTIBIOTIC THERAPY

Tetracycline and chloramphenicol are effective.

Table 20.2: Weil-Felix reaction.

GROUP	OX 19	OX 2	OX K
Typhus group	+++	-	-
Scrub typhus	-	-	+++
Spotted fever group	+	+	-

MYCOPLASMA

They are classified as bacteria but differ from them the in following:

1. They are smallest of all the free-living bacteria, having a size of 125-250 nm.
2. They lack a rigid cell wall and have a cytoplasmic membrane containing sterols.
3. Due to deficient cell wall their shapes vary from cocci to long filaments, and are highly pleomorphic.
4. They do not stain with routine bacterial stains.

SPECIES OF MEDICAL IMPORTANCE

The genera of the order Mycoplasmatale are Mycoplasma, Ureaplasma, Acholeplasma, Spiroplasma and Anaeroplasma. Medically important species are *Mycoplasma pneumoniae, Mycoplasma hominis* and *Ureaplasma urealyticum.* The Mycoplasma are found freely in the soil, air and in animals.

MORPHOLOGICAL CHARACTERISTICS

These are not visible under light microscope in clinical specimens because of very small size, but can be seen in cultured growths. They do not stain with Gram stain because of deficient cell wall but can be seen by dark ground microscopy and by immunofluorescence as signet rings, cocci, bacilli and filaments.

CULTURAL CHARACTERISTICS

Special medium for *Mycoplasma* is mycoplasma agar containing meat infusion peptone broth, 30% human ascitic fluid, horse, or rabbit serum. Incubated at 37°C. Growth appears between 3-10 days as very small colonies only visible by a lens. The contours are round with a dark centre buried in the medium and edges are thin. It is called inverted fried egg appearance. The growth occurs under microaerophilic conditions.

PATHOGENICITY

M.pneumoniae causes atypical pneumonia. *M.homonis* can cause pelvic inflammatory disease or puerperal fever in females. *U.urealyticum* is urease positive and causes

non-gonococcal urethritis in men.

SEROLOGY

A four-fold increase in titre or a single high titre is indicative of disease. Antibodies are detected by complement fixation, immunofluorescent, cold-agglutinin (nonspecific but may be helpful) and by radioimmunoprecipitation, complement dependent cidal assay and colony inhibition on agar.

ANTIBIOTIC SENSITIVITY

Mycoplasma are resistant to all antibiotics that act on cell wall e.g., penicillin and cephalosporins. They are sensitive to tetracyclines and erythromycin.

LEGIONELLA

Legionella pneumophila is a thin, Gram-negative rod. It tends to display pleomorphism. They do not stain well in tissues with the Gram stain. Silver impregnation stains are the most useful. *L.pneumophila* does not grow on routine bacteriological medium without the addition of cysteine and ferric ions, colonies on agar medium have a ground glass appearance. They require 3-5 days for growth at 35°C under aerobic conditions.

21. EXAMINATION OF CLINICAL SPECIMENS

Collection and transport of clinical specimens have already been described in detail (see COLLECTION OF SPECIMEN on page 68). Further processing and handling of microbiological specimens is discussed here. A microbiology laboratory is responsible to deal with the specimens for culture. The general guidelines are:

1. Check the specimen and request form for labelling error. Ensure that specimen and request form correspond with each other and are from same person.
2. Different specimens from same patient are dealt separately. All specimens are kept properly till they are processed e.g., urine has to be kept at 4°C, while CSF is to be kept at 37°C (If the specimen is for bacterial and not for virual culture).
3. If specimen is for culture, then make a direct slide for Gram stain. If two swabs are received, one is used for making slide and other for culture. In case only one swab is received, culture is put up before making the slides for staining.
4. The selection of media and their incubation depends upon the suspected pathogen, e.g., in case of CSF; MacConkey agar is put up for Gram-negative bacilli and Chocolate agar for *Neisseria meningitidis* and pneumococci.
5. All specimens from sites having normal flora will also yield growth of normal commensals. In certain situations early reporting is of clinical importance e.g., in meningitis. In such situations primary or direct sensitivity i.e., on the clinical specimen can be performed, so as to get the antimicrobial susceptibility results within 24 hours.

EXAMINATION OF FAECES AND RECTAL SWABS

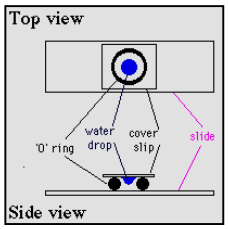
PHYSICAL EXAMINATION

This is similar to the one described for EXAMINATION OF FAECES on page 89.

MICROSCOPIC EXAMINATION

Other than a serarch for parasites, this is not usually done, but occasionally lab may be requested to look for pus cells.

1. **Examination of wet film**: This has already been described in section on DIRECT WET PREPARATION on page 90.
2. **Methylene blue staining**: This is required to demonstrate the pus cells in stool specimen. Follow the above procedure except that in place of saline take a drop of methylene blue stain.
3. **Gram staining**: Gram stain is required in suspected infections with Campylobacter, clostridium, candida or other fungi. Campylobacter may be seen as Gram-negative curved rods. Clostridium spp. may be seen as Gram-positive rods and if they are completely filling the field, then they are significant. Similarly Candida spores can be identified.
4. **Motility**: It is done directly on stool specimen if *Vibrio cholerae* is suspected. It is performed by **hanging drop** method from specimen itself or alkaline peptone water if the specimen is brought in it. If organism is found to be motile, showing darting motility; then repeat the motility with a drop of *Vibrio cholerae*-O antiserum. If the organisms are immobilised then provisional diagnosis of *Vibrio cholerae* can be made. A welled slide is best used for this purpose. A drop of faecal suspension is placed in the centre of a cover slip and is inverted over the well. Margins of drop are examined under the microscope with closed aperture and diaphragm pulled down to give good contrast.



CULTURE

Day-0: Make a suspension of formed stools in 1 ml peptone water or loose stools can be inoculated as such on MacConkey agar, Deoxycholate Citrate Agar (DCA) or Xylose Lysine Deoxycholate Agar (XLD) [usually two enrichment broths are used], Tetrathionate (TT) broth and Selenite F (SF) broth. In addition, put up a culture on blood agar. This is required if the patient is <5 years of age for *Escherichia coli* agglutination. *Campylobacter* selective medium is used if required. In a suspected case of cholera a culture in Thiosulphate Citrate Bile

Salt Sucrose medium (TCBS) and Alkaline Peptone water is performed. From alkaline peptone water subcultures are made on fresh alkaline peptone water and TCBS medium after 6 hours. All the media are incubated aerobically at 37°C for 18-24 hours except *Campylobacter* medium, which is incubated at 42°C in anaerobic jar. It is occasionally recommended that an ordinary anaerobic gas generating kit be used without putting a catalyst in the jar but it is inadequate for culturing campylobacters. If special microaerophilic gas generating kit (Campy Pak) is not available, candle jar (5-10% CO_2) can be used. If *Yersinia* is suspected then MacConkey agar plate is incubated at 20-28°C.

Day-1: All the plates are examined for growth. Look for non-lactose fermenting (NLF) colonies on MacConkey and DCA agar. Most of the enteric pathogens give NLF (pale) colonies. *Proteus* are abundant in the gut and *Pseudomonas* that may be present in stool but are non pathogenic in gastrointestinal tract also give a non-lactose fermenting growth. Following tests are put up and results are noted immediately or within 1-4 hours.

1. Oxidase test (for exclusion of *Pseudomonas* but one should note that *Vibrio cholerae* is also oxidase producer)
2. Urease test (for exclusion of *Proteus*)
3. Indole test (for exclusion of *Escherichia coli* in case patient is >3 years of age).

If above tests are negative then it is dealt as pathogen and these NLF colonies are identified by usual procedure of Gram staining, motility testing and biochemical tests (Sugar sets) followed by antibiotic sensitivity testing. In case of a child, the growth from the blood agar is used for *Escherichia coli* agglutination by the antisera of the diarrhoeagenic strains. In case, there are no NLF colonies, sub-cultures from TT broth and SF broth on MacConkey agar and DCA or XLD agars are done and examined for NLF colonies. If found, they are dealt as described above. Examination of the plate of *Campylobacter* is done after 72-96 hours. If there is a growth then proceed with identification. Campylobacter are oxidase positive. On TCBS agar yellow colonies are looked for which are sub-cultured on the blood agar for further identification. If MacConkey agar is kept at room temperature and it shows small non-lactose fermenting colonies then proceed for identification of *Yersinia enterocolitica*. It is essential that when picking colonies for further identification, only isolated single colonies be chosen. If purity cannot be guaranteed, subculture the colony, remember that correct processing needs care rather expertise.

Day-2: Read the biochemical reactions, make the identification, and if the organism is an enteric pathogen then report it with its sensitivity.

LIST OF ENTERIC PATHOGENS

1. *Salmonella* spp.
2. *Shigella* spp.
3. Diarrhoeagenic *E. coli* (for details see *ESCHERICHIA COLI* PATHOGENICITY on page 136).
4. *Vibrio cholerae*, and
5. *Vibrio parahaemolyticus*
6. *Campylobacter* sp
7. *Yersinia enterocolitica*
8. *Clostridium perfringens* (Type A and C)
9. *Clostridium difficile*
10. *Helicobacter pylori* (culture is not always required).

EXAMINATION OF PUS

GROSS EXAMINATION

The following characteristics are to be noted:

- Colour: Pyocyanin and other pigments, or blood. Chocolate brown in amoebic infections (page 115), greenish in *Pseudomonas* infections (page 140).
- Consistency: Thin and watery or thick and purulent. Cheesy pus may be due to *Mycobacterium tuberculosis* (page 146).
- Smell: Many anaerobes give foul odour
- Presence of granules: Yellowish granules are usually from the pus of mycetoma due to *Actinomyces* spp (page 135).
- Fluorescence in long wave (UV) light: *Provotella melaninogenica* (usually useful on pus from brain or lung abscess).

MICROSCOPIC EXAMINATION

The commonest problem with making films is that material tends to float or lift off the slide during staining. The following tips may help:

- Gently warm the slide first.
- Use a swab rather than a loop to apply the pus.
- Keep the smear thin.

Examine the fresh pus in a drop of saline under x40 objective for amoebic vegetative forms if amoebic abscess is suspected. Take a small portion of the pus in sterile distilled water and shake it. Now let it settle down. With the pasture pipette, transfer the sediment on a slide and perform Gram stain and Ziehl-Neelsen staining.

CULTURE

Day-0: In case the pus is from the site below the diaphragm, cultures are made on blood and MacConkey agar, and are incubated aerobically at 37°C and on neomycin or gentamicin blood agar for anaerobic incubation at 37°C. On this plate a disk of metronidazole is placed for identification of anaerobes. If anaerobic jar is already loaded, anaerobic cultures can also be made on Robertson cooked meat medium (RCM) to economise. Subculture from RCM is made on anaerobic blood agar next day. If the specimen is from above the diaphragm or isolation of Haemophilus spp. or *Streptococcus pneumoniae* is to be done, culture on chocolate medium is made. Lowenstein Jensen medium is inoculated if tuberculosis is suspected. If actinomycosis is suspected and granules are not available, a 1 in 10 to 1 in 100000 dilution is inoculated on blood agar for incubation in 5-10% CO_2, two blood agar plates for anaerobic culture (one for 48 hours and other for 7 days), thioglycollate broth, RCM and 1% glucose semi-solid agar. All are incubated at 37°C. In addition, selective medium containing colistin (10 mg/L), kanamycin (7.5 mg/L), metronidazole (2.5 mg/L), nalidixic acid (15 mg/L), vancomycin (100 mg/L) and phenyl ethyl alcohol 25% are used. Occasionally, bacteria seen on a Gram film fail to grow due to the presence of antimicrobial substances (usually antibiotics). By far the commonest type of specimen received is a **wound swab** from soft tissue infection. Microscopy takes second place after culture unless more than one swabs are received from the same site. Swabs are not suitable for examination of mycobacteria.

Day-1: Examine the culture plates and RCM for blackening or reddening and for gas, make slides for Gram stain and subculture on appropriate media. Identify the organisms grown by catalase, oxidase, coagulase, motility and other biochemical tests. Simultaneously antimicrobial sensitivity is put up.

Day-2: Identify the organisms and report with their antimicrobial susceptibility. L-J media needs incubation for 4-6 weeks and is examined weekly for any growth. Similarly for actinomycosis plates are examined after 2 and 7 days.

COMMON ORGANISMS ISOLATED FROM PUS

1. *Staphylococcus aureus*
2. *Streptococcus pyogenes*
3. *Enterococcus faecalis*
4. *Escherichia coli*
5. *Klebsiella pneumoniae*
6. *Citrobacter freundii*
7. *Enterobacter cloacae*
8. *Pseudomonas aeruginosa*
9. *Clostridium* species
10. *Bacteroides* species

EXAMINATION OF URINE

PHYSICAL EXAMINATION

As described in section on Urine Examination (page 77).

MICROSCOPIC EXAMINATION

Microscopy of urine may be performed on centrifuged or uncentrifuged urine. Microscopy of uncentrifuged, unstained urine by microtitre tray and inverted microscope method or a disposable counting chamber are the commonest methods used. Examine a wet preparation as described in 'Urine Examination' on page 84. For *M. tuberculosis* culture, about 100-200 ml of urine is centrifuged in 4-5 large test tubes, deposits are mixed in one tube and are re-centrifuged, and smears are made from deposit and stained with Ziehl Neelsen methods.

CULTURE

Semi-quantitative urine cultures: It can be done by calibrated loop technique, paper foot method (Bacteriuritest strip) or by multipoint inoculation method. The urine culture can be quantitative or semi quantitative, when bacteria per ml of urine are estimated. If the estimated number of bacteria in urine is $<10^4$, then it is not an infection but are because of contamination by urethral commensals. If the number is between 10^4 and 10^5 then there can be due to infection or contamination. If the number comes to $>10^5$ per ml, then infection is presumed. However, in certain special conditions lesser number of microorganisms may be significant e.g., pregnancy, immunocompromised and patient on antibiotics etc.

Procedure:

Day-0:

- A 3 mm loop that picks up a standard amount of urine is used. The tip of loop is dipped into the urine to pick only the required amount of urine. This is inoculated on blood and MacConkey agar and incubated aerobically at 37°C.
- Alternatively only one plate of CLED medium can be inoculated quantitatively.
- If loop is calibrated to pick 0.01 ml of urine and 30 colonies appear on the plate then

bacterial count will be 30x100=3,000 bacteria/ml. In other method a filter paper strip that carries the standard amount of urine is dipped in the urine up to the mark and are inoculated on CLED (Cysteine lactose electrolyte deficient medium) or MacConkey agar. The strip picks 0.2 µl urine. If there are 20 colonies on the inoculated area it gives a count of 10^5 bacteria/ml.

Quantitative urine culture is required in following conditions:

a. If *M. tuberculosis* is to be isolated.
b. If *Salmonella* spp. is to be isolated.
c. If any specific organism is to be isolated as a cause of any outbreak or for any other reason.

If renal tuberculosis is suspected, three early morning specimens are collected and kept refrigerated or if patient has to come from far off area or because of any logistic problem 24 hours urine sample can be collected. The supernatant is discarded and the sediment is centrifuged and inoculated after decontamination by Petroff's method on L-J medium.

Day-1: The culture plates are examined for growth and read as usual. The significant colonies are identified (biochemical tests etc.) and antimicrobial sensitivity is put up. If there is no growth, the culture plates are re-incubated for further 18-24 hours.

Day-2: Results of identification and sensitivity tests are reported.

EXAMINATION OF CEREBROSPINAL FLUID

CSF examination is an emergency and positive findings are to be communicated to the treating physician immediately.

PHYSICAL EXAMINATION

It is done as described in EXAMINATION OF CEREBROSPINAL FLUID (CSF) on page 94.

MICROSCOPIC EXAMINATION

Slides are made from the centrifuged deposit of CSF (1800g for ten minutes or by cytocentrifuge if available) and stained with Gram, Leishman and Ziehl-Neelsen methods. These are then examined for microorganisms and type of cells (page 95). Supernatant is observed for xanthochromia (page 94). A drop of CSF is mixed with India ink to look for *Cryptococcus neoformans*. These are seen as large, round, bodies of 5-22 µm size, stained with India ink and around them are a large unstained capsule seen as a halo. A wet preparation is examined for amoebae and trypanosomes if required.

CO-AGGLUTINATION TEST FOR BACTERIAL ANTIGENS

Sometimes immediate identification of microorganisms is required for instituting appropriate therapy as cultures may take longer and ultimately fail. These tests are thus needed in emergency for quick diagnosis particularly when patient has taken antimicrobials. This is done by specific serological kits. CSF is boiled in water bath for 5 min and centrifuged. The supernatant is tested for Streptococcus group B, Haemophilus influenzae type b, *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Escherichia coli*, Cryptococcus *neoformans* and Candida spp.

CULTURE

Day-0: CSF is inoculated as soon as it is received. If delay is anticipated in processing, it should be kept at 37°C in an incubator and **should never be placed in refrigerator**. It is cultured on chocolate agar (for Neisseria meningitidis, *Streptococcus pneumoniae* and Haemophilus spp.*)* and MacConkey agar (for Gram-negative bacilli). Chocolate agar is incubated in a candle jar at 37°C (5-10% CO_2) and MacConkey agar aerobically at 37°C. If tuberculosis meningitis is suspected, inoculate L-J medium and if Cryptococcus neoformans or other fungi are suspected then inoculation on Sabouraud's agar and blood agar at 37°C aerobically. A primary sensitivity test on chocolate agar is also incubated at 37°C in a candle jar.

Day-1: Plates are examined and any growth obtained is dealt with for identification and sensitivity. If there is no growth, the culture plates are re-incubated for another 24 hours. *M. tuberculosis* and fungi may require long incubation.

Day-2: Report the results of identification and sensitivity test.

EXAMINATION OF SPUTUM

PHYSICAL EXAMINATION

Testing the expectorated sputum is the only non-invasive method of examining the lower respiratory tract secretions in various diseases. It is a simple but one of the most common specimen sent to a laboratory. In order to be meaningful, it is important that the quality of specimen collected is of standard. Sputum can be purulent like pus, muco-purulent (pus and

mucus mixed), mucoid (mucus only) or muco-salivary (mucus in saliva). If only saliva is received, it is unfit for culture. Note the colour; yellowish (tuberculosis) rusty (pneumonia), greenish (*Pseudomonas* infection) or chocolate (amoebic abscess).

MICROSCOPIC EXAMINATION

Make a wet preparation and look for epithelial cells. Presence of more epithelial cells then pus cells indicates poor collection and makes the specimen unsatisfactory, unfit for culture. (The ratio of pus cells and epithelial cells should be more than 10:1). However, the following are exceptions:

a. Neutropenic patient
b. Immunocompromised patient
c. Endobroncheal wash
d. Tracheal aspirate

Make thin smears from purulent part and heat-fix in a class-I safety cabinet before staining with Gram and Ziehl-Neelsen methods. Normally the sputum contains many Gram-positive and Gram-negative organisms added to it from the normal flora of the upper respiratory tract. Look for likely pathogens and those in abundance like pneumococci*, klebsiella, haemophilus* etc. In Ziehl-Neelsen stained smear look for AFB.

CULTURE

Day-0: The sputum is first homogenised to reduce within specimen sampling error. Alternatively, some laboratories only opt to pick the purulent fleckes of sputum. The sputum is cultured after washing with saline or treating it with a liquefying agent (sputolysin). Semiquantitative cultures have been used in patients with cystic fibrosis. One technique is that sputum is inoculated after 1 in 1000 to 1 in 10,000 dilution. If there are pathogens in 1 in 10,000 dilution then they are significant. Ten µl-diluted sputum is cultured on blood agar and chocolate agar aerobically at 37°C). If the patient is immunocompromised or if nosocomial infection is suspected, MacConkey agar is also inoculated. The plates are incubated for 18-24 hours. Optochin disc (5 µg) is placed on the chocolate agar plate. Decontaminated and homogenised sputum is inoculated on L-J medium if pulmonary tuberculosis is suspected.

Decontamination of sputum and other material (Petroff's Method)

1. Transfer 1-2 ml specimen to a test tube/universal container.
2. Add equal amount of 4% NaOH.
3. Incubate at 37°C for 30 min, mixing and shaking at intervals.
4. Centrifuge at 1500g for 30 min.
5. Discard the supernatant.
6. Add a drop of phenol red and neutralise the deposit with 8% HCl drop-by-drop till it just become pink.
7. Transfer 2-3 drops of deposit to a Lowenstein Jensen slope.
8. If acid L-J medium is available, the step 6 can be omitted and 2-3 drop of deposit can be inoculated on it.

Day-1: Examine blood agar and chocolate agar plates for pure growth of *Streptococcus pneumoniae*, *Haemophilus influenzae, Streptococcus pyogenes, Klebsiella pneumoniae* and *Staphylococcus aureus*. If the number of colonies is more than 10 in dilution of 1000, the number of organisms is more than 10^6/ml of sputum. The count of the microorganisms should be more than 10^6/ml or deal any organisms, which is found as pure growth. The organisms grown are dealt for identification and sensitivity. The optochin disc on the chocolate agar plate will help in the identification of *Streptococcus pneumoniae*, which is optochin sensitive. In case no significant growth is obtained the culture plates are re-incubated for another 24 hours.

Day-2: The organisms are reported with their sensitivity pattern.

EXAMINATION OF THROAT SWABS

MICROSCOPIC EXAMINATION

Infections of the throat may be bacterial or, more commonly, viral in origin. Commonest bacterial infection of throat is due to *S.pyogenese* (Lancefield group A haemolytic streptococci). Group C and G can also cause pharyngitis. *C.diphtherie*, *C.ulcerans* and *A.haemolyticm* are the other pathogens. Smears are made and stained with Gram and Albert methods. Look for pus cells and Vincent's organisms, which are Gram-negative spiral rods. Sometimes Gram stain reveals large spores of Candida spp. in patients on broad-spectrum antibiotics or in immunocompromised patients. On Albert stained smear identify Corynebacterium diphtheriae if diphtheria is suspected. They are greenish rods with dark purplish granules at poles. They are of different sizes and show palisade arrangement. If a clinician has asked for Albert staining (commonly known as KLB staining) the report whether negative or positive should immediately be communicated.

CULTURE

Day-0: Throat swabs are cultured on blood agar and Tellurite Blood Agar (TBA) [for Corynebacterium diphtheriae] the plates incubated aerobically at 37°C. On the blood agar plate a bacitracin disc is also put up. Loeffler's serum is inoculated for Corynebacterium diphtheriae. The growth from this semi-solid medium is used for Albert staining and subculture on blood as well as on Tellurite blood agar after 6 hours of incubation at 37°C. Detection of *S.pyogenes* can be enhanced by anaerobic culture for 48 hours.
Day-1: Examine the culture plates. Group A, β-haemolytic streptococci are sensitive to bacitracin. Identify the organisms and perform detailed antimicrobial susceptibility testing. On Tellurite blood agar black colonies could be of *Corynebacterium diphtheriae*, diphtheroids and Staphylococci. Any growth on TBA should be identified by Gram and Albert stain. If Corynebacterium like organisms are present, put up the Hiss's sugar set for identification and antimicrobial sensitivity. Examine the plates and sugars set and prepare report the next day..

EXAMINATION OF NASAL SWAB

MICROSCOPIC EXAMINATION

Prepare smears, stain with Gram stain and examine microscopically.

CULTURE

Day-0: Inoculate on blood agar and incubate aerobically at 37°C and Chocolate agar is incubated at 37°C with CO_2. In suspected whooping cough case additional medium for *Bordetella pertussis* is inoculated (Charcoal Cephalexin Blood Agar) (CCBA).
Day-1: Examine the blood agar plate for β-haemolytic colonies of *Streptococcus pyogenes*. This is done to detect the nasal carriers of these organisms. Examine chocolate agar for colonies of *N.meningitidis*, *H.influenzae*, *Staphylococcus aureus* and *Streptococcus pneumoniae*. If any of these organisms is suspected, proceed for identification and sensitivity. If no significant growth is seen, re-incubate all the culture plates.
Day-2: Identification and sensitivity test are reported..

EXAMINATION OF EAR SPECIMEN

MICROSCOPIC EXAMINATION

Prepare smears, stain with Gram stain and examine microscopically. If fungal infection is suspected prepare smears from swab in KOH or saline and examine for fungal spore and hyphae.

CULTURE

Day-0: The swab or pus is inoculated on Blood and MacConkey agar and incubated aerobically at 37°C. If the patient is a child, chocolate agar is also inoculated and incubated at 37°C with 5-10% CO_2. If a chronic ear infection is present anaerobic blood agar is inoculated and incubated anaerobically at 37°C.
Day-1: Examine the culture plates for growth. Prepare Gram stained smears, examine morphology and put up identification and antimicrobial sensitivity tests.
Day-2: Read identification and sensitivity tests and prepare report.

COMMON EAR PATHOGENS

1. Pseudomonas species
2. Proteus species
3. *H.influenzae* (especially in children)
4. *Staphylococcus aureus*
5. *Streptococcus pneumoneae*
6. β-haemolytic streptococci
7. Candida species
8. *Aspergillus* species
9. *Bacteroides* species

EXAMINATION OF EYE SPECIMEN

MICROSCOPIC EXAMINATION

Prepare the smear and stain with Gram stain. Examine the smear under the microscope for bacteria and pus cells. In neonates particularly look for *Neisseria.*

CULTURE

Day-0: The swabs are inoculated on Blood agar, incubated at 37°C aerobically and on Chocolate agar incubated in the presence of 5-10% CO_2 at 37°C.
Day-1: Examine the plates for growth and identify. If required put up identification and sensitivity tests.
DAY-2: Read identification and sensitivity tests and prepare report.

EXAMINATION OF FLUID ASPIRATES

PHYSICAL EXAMINATION

As for CSF see page 157.

MICROSCOPIC EXAMINATION

Examine Gram stained smears for organisms

and Z-N stained smears for AFB as on kpage 157. A positive Gram stain result needs to be passed on to treating physician by telephone.

CULTURE

Day-0: Proceed as for CSF (see page 157). Inoculate the sediment on Blood and MacConkey agar to incubate aerobically at 37°C and Chocolate agar to incubate in CO_2 Jar at 37°C. Anaerobic Blood agar is also inoculated and incubated at 37°C anaerobically. L-J medium is inoculated if tuberculosis is suspected.

Day-1: Examine all the plates after 18-24 hours incubation. The anaerobic plate is kept for 48-72 hours. If there is any growth, identify it and put up the sensitivity. L-J slope is kept for 6-8 weeks and examined weekly for growth.

Day-2: Read identification and sensitivity tests and prepare report.

BLOOD AND BONE MARROW CULTURES

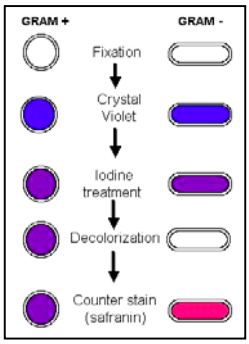
Use of two media is preferred as it increases the chances of isolation. Trypticase Soya broth and Brain Heart Infusion (BHI) broths are commonly used. Thioglycollate broth is used for anaerobic microorganisms. The bottles are incubated at 37°C and examined daily for visible turbidity. These are subcultured on day 1,2,4 & 7 on blood and MacConkey agar. Gram smears are also preparesimultaneously to see any visible growth. The bottles are usually kept for 7 days except for brucellosis and endocarditis, where these are incubated for 4-6 weeks. The bottles are incubated in CO_2 containing atmosphere for Brucella. (It is essential to loosen the caps of bottles during incubation). Identification and sensitivity tests are put up If the growth appears on the subculture. These are read the next day and reports are prepared. The bottles showing no growth are discarded after 7 days except in suspected case of *Brucella* culture. Dealing with blood cultures requires strict aseptic technique, right from the collection of blood to the sub-cultures. There is a high risk of introducing organisms from out side. To avoid this following procedures are available:

1. Manometric signal system blood culture bottles of special shape are now available for simplicity. There is an upper chamber above the bottle containing media. Whenever, there is growth in the medium this chamber gets filled and from here the Gram smears and sub-cultures can be made.
2. The diphasic Castaneda system avoids the problem associated with frequent sub-culturing. The device consisting of clear plastic screw capped bottle with an internal paddle or dipstick holding sterile medium. After addition of patient's blood, the screw cap is removed and replaced with this assembly. The blood culture bottle is then transiently inverted so that the contents flow over the medium and the whole assembly is incubated. The inversion can be repeated once or twice daily. The growth can be visible on the surface of the solid part of the medium.
3. Automated system for blood culture is also available e.g., Bact Alert (see page 59). In this system subculture is not required. The device itself indicates through light signal if there is any growth.
4. Lysis – centrifugation method is better than conventional and radiometric methods for detection of fungi and mycobacteria.

22. STAINING PROCEDURES

GRAM STAINING

Principle: Christian Gram originally described this stain in 1884. The mechanism of Gram staining is not fully understood. Gram-positive bacteria stain with crystal violet and are not decolourised with acetone iodine, while Gram-negative bacteria are decolourised with acetone iodine and hence take up the colour of the counter stain (carbol fuchsin). The difference in staining is due to the difference in the cell wall structure. Gram-positive bacteria have thick layer of peptidoglycan in their wall while Gram-negative bacteria have a thin layer. The original technique has undergone many modifications and the most widely used is the Preston and Morrell's modification, which is described below.



Reagents

1. Ammonium oxalate crystal violet solution

Crystal violet	20 g
Methylated spirit	200 ml
Ammonium oxalate 1% aqueous solution	800 ml

2. Iodine solution

Iodine	10 g
Potassium iodide	20 g
Distilled water	1000 ml

3. Iodine acetone

Liquor iodi fortis	35 ml
Iodine	10g
Potassium Iodide	6g
Methylated spirit	90 ml
Distilled water	10 ml
Acetone	965 ml

4. Dilute carbol fuchsin

Carbol fuchsin (page 161)	50 ml
Distilled water	950 ml

Procedure: Make a thin smear, dry in air and fix in flame. Cover with crystal violet for 30 seconds. Wash and apply iodine solution for 30 seconds. Wash and decolourise with acetone iodine until no further violet washes off. Wash and counterstain with dilute carbol fuchsin for 30 seconds. Wash with water, blot and dry.

Result

Gram-positive bacteria	Dark purple
Yeast cells	Dark purple
Gram-negative bacteria	Pale to dark red
Nuclei of pus cells	Red/pinkish
Epithelial cells	Pale red/pinkish

Interpretation: The report should include the following information:

1. The number of bacteria (numerous, moderate, few or scanty)
2. The Gram reaction (Gram-positive or Gram-negative)
3. The morphology (cocci, intracellular or not)
4. The presence and number of pus cells
5. Presence of yeast or epithelial cells

Findings: Gram stain of urethral smear shows numerous pus cells and moderate number of Gram-negative diplococci, some of which are intracellular. Similarly, Gram stain of sputum may show numerous pus cells with a few epithelial cells and a moderate number of Gram-positive cocci in chains and a few Gram-negative bacilli are present.

Variations: Gram-positive organisms may lose their ability to retain crystal violet and stain Gram negatively for the following reasons:

1. Cell wall damage due to antibiotic therapy or excessive heat during fixation.
2. Over-decolourisation of the smear.
3. Use of an old Iodine solution (yellow instead of brown). It is to be stored in brown bottle.
4. Old culture

Quality Control: Known Gram-positive (*Staphylococcus aureus*) and negative (*Escherichia coli*) organisms can act as controls on the same slide.

ZIEHL-NEELSEN STAINING

Principle: The technique is used to stain *Mycobacterium* and *Nocardia* species. Mycobacteria when stained with carbol fuchsin resist decolourisation by acid while other bacteria get decolourised. Hence these are called Acid Fast Bacteria (AFB). *Mycobacterium leprae* is less acid fast and is decolourised with 5% acid. Similarly *Nocardia* species and *Legionella* species are even lesser acid fast and 1% acid is used for decolourisation.

Reagents

1. Carbol fuchsin (mordant and dye): Basic fuchsin solution (10 g in 100 ml 95% ethyl alcohol) to be added to 900 ml 5% aqueous solution of crystalline phenol (see also MODIFIED ZIEHL-NEELSEN STAIN on page 393)

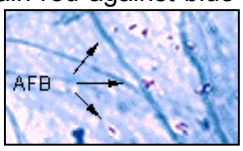
2. Acid alcohol (decolourising agent)
   a. Sulphuric acid 20 percent
   b. Ethyl alcohol 95%
3. Counter stain, Loeffler's methylene blue (saturated solution of methylene blue in alcohol 300 ml).

Potassium hydroxide, 0.01% in water up to 1000 ml.

Procedure: Fix smear by rapidly passing over flame. Cover with filtered carbol fuchsin and heat until steam rises. Allow staining for 5 min, heat being applied at intervals to keep the stain hot. Do not boil or allow to dry. Wash with water. Decolourise with acid alcohol for 2 min. The red colour of the preparation changes to yellowish brown. Wash with distilled water and counter-stain with Loeffler's methylene blue for 15-20 seconds. Wash in distilled water, dry and examine.

Results: Acid Fast Bacilli stain red against blue background.

Interpretation: If bacilli are seen, report as "AFB positive" or "Acid Fast Bacilli seen". To report the result quantitatively as bacterial index the following chart is used:



No per entire smear	Significance
1-2	Doubtful (repeat)
3-9	Rare (1+)
≥10	Few (2+)
≥1 (per oil immersion field)	Numerous (3+)

Control: Two sputum smears of known high and low AFB positivity should be stained with the routine smears to check the procedure and interpretation at regular intervals and whenever a new batch of stain is introduced.

Modifications: Modifications of Ziehl-Neelsen staining method for other acid-fast organisms are:

1. For *Mycobacterium leprae* decolourisation is done with 5% sulphuric acid (or 3% HCl in alcohol).
2. Sections of tissue containing 'clubs' of *Nocardia* and *Legionella* are decolourised with 1% sulphuric acid.
3. Cultures of some specimens of Nocardia are decolourised with 0.5% sulphuric acid.
4. Stool specimen for Cryptosporidium parvum are decolourised with 3% hydrochloric acid or 10% sulphuric acid.
5. Auramine phenol method has the advantage that large areas of film can be stained in a short time.
6. When heat is not required Kinyuon method of cold acid fast staining can be used. In this modification the Initial step of heating carbol fuchsin is omitted. Instead the stain is put up on the smear for longer period (20-30 min).

ALBERT'S STAINING

This stain is used to identify Corynebacterium diphtheriae *and to* stain the volutin (metachromatic) granules.

Reagents

1. Solution 1 (Albert's stain): Dissolve 0.15 g Toluidine blue and 0.2 g Malachite green in 2 ml 95% ethyl alcohol, add 100 distilled water and 1 ml glacial acetic acid. Mix well, let stand for 24 hours and filter before use.
2. Solution 2 (Albert's iodide): Grind 2 g Iodine crystals and 3 g Potassium iodine in about 10 ml distilled water and make volume to 300 ml with distilled water.

Procedure: Allow smear to dry and fix by heat. Stain with solution 1 for 1-5 min. Rinse with water and blot dry. Apply solution 2 for 1 min. Rinse with saline, blot dry and examine.

Result: Bacterial cells stain green and volutin granules stain green black.

Control: Smear of a positive control and negative control on the same slide are made for comparison.

SPORE STAINING

Principle: The wall of spores is relatively impermeable, but dyes can be made to penetrate it by heating, once stained, these resist decolourisation.

Reagents

1. Ziehl Neelsen's carbol fuchsin.
2. Sulphuric acid 0.5%
3. 1% aqueous methylene blue or 5% aqueous malachite green.
4. 5% safranin or 0.05% basic fuchsin.

Acid Fast Stain for Spores

Procedure: Make film, fix, dry and stain with for 3-5 min with heat. Wash in water and decolourise with 0.5% sulphuric acid. Wash with water and counterstain with 1% aqueous solution of methylene blue for 3 min. Rinse in water, blot and dry, and examine.

Malachite Green Staining

Procedure: Make smear, fix and dry. Place slide over a beaker of boiling water. When large drops of water condenses on the under side of slide, flood it with malachite green and leave for 1 min. Wash in cold water. Counterstain with 0.5% safranin or 0.05% basic fuchsin for 30 seconds. Wash, dry and mount.

Interpretation: By Ziehl-Neelsen stain spores appear bright red while protoplasm stains blue. With malachite green spores stain green while vegetative bacilli stain red.
Result: Report as presence or absence of "spore forming bacteria". Specify position of spores (terminal, sub-terminal or central) and also number of spores per bacteria.
Control: Control should be included in the smear to assess reagent's efficacy.

GIEMSA STAINING

It is one of the Romanowsky stains described in haematology (page 258). These stain cytoplasm as blue and nuclei as red.
Reagents: Stock Giemsa stain: Grind 3.8 g Giemsa stain powder in 200 ml glycerine. Place it at 60°C for 2 hours. Cool and add 312 ml methanol.
Procedure: Prepare smear and dry. Fix in methanol for 3-5 min and dry in air. Place in 1:10 diluted Giemsa stain[1] for 30-45 min. Sodium carbonate (1%) can be used as diluent instead of distilled water. Spirochetes may require up to 4 hours. Wash with water, blot dry and examine.

INDIA INK STAINING OF BLOOD AND CSF

India ink is useful for visualisation of capsulated organism such as Cryptococcus neoformans (page 197) or *B. anthracis* in clinical samples or from fungal cultures.
Procedure: Take 100 µl of sample (blood, CSF, suspension of fungus culture) or control to a slide, place a cover glass and add 5-10 µl India ink to the edge of the cover glass. The preparation is ready for examination.
Control: *Klebsiella pneumoniae* on SBA or equivalent or known *C.neoformans* are used as positive control. *E. coli* ATCC 25922 or equivalent act as negative control. Transfer a small amount of growth (1 mm diameter) from each control SBA plate into 0.5 ml whole EDTA-treated blood or serum and mix. Control strains are assayed on each day of testing.
Interpretation: The capsule will appear as a well-defined clear zone around the cells for the positive control. No zone should be present in the negative control.

MCFADYEAN'S STAIN

This is a special stain for the capsule of *Bacillus anthracis*.
Procedure: Make a thick smear of blood, exudate or tissue fluid, dry in air and fix imperfectly by passing quickly three times through a flame. Stain with polychrome methylene blue for 30 seconds. Wash and dry.
Interpretation: Irregular pink-purple capsular material, both surrounding the bacilli and chains of bacilli and some detached from them, is indicative of anthrax bacilli.

PHENOL-AURAMINE STAIN

This stain is used for detection of *cryptosporidium* oocyst. It gives consistent results with few false positives. The oocyst shows a bright centre with inclusions and a pale halo, when examined with blue light under a fluorescent microscope.

[1] It is preferable to pour stain in a container like a Petri dish or Coplin jar and place the slide in an inverted position, because this avoids stain deposits on the slide.

23. PREPARATION OF CULTURE MEDIA

It is essential for identification of bacteria to obtain a culture by growing the organisms on artificial media. If more than one species or types are present then sub-cultures are required. The preparation of suitable culture medium entails following important steps:

1. The preparation of a culture medium.
2. Sterilisation of the media.
3. Adjustment of pH.

PREPARATION OF MEDIA

The basis for almost all the bacteriological media is meat extract (broth) providing most of the required nutrients. Commercial meat extracts such as **Lab Lemco** is also available. Other growth requirements of bacteria are provided by digested and un-coagulable proteins to the broth in the form of commercial peptone. The media may be solid or liquid. The solid media are prepared by addition of gelatin or agar to the broth to a final concentration of 1-2%. Gelatin is an albumin like material derived from bones, tendons and cartilage. Agar is obtained from dried seaweed. Eggs and potatoes can also be used to solidify liquid media.

TYPES OF CULTURE MEDIA

1. Simple media
2. Enriched media
3. Selective media
4. Differential media
5. Enrichment media

Simple Media: These contain basic nutrients for bacterial growth like broth with peptone with or without solidifying agent. Examples are nutrient broth and peptone water.

Enriched Media: Simple media are sometimes not appropriate for the isolation and subsequent growth of pathogenic bacteria. It thus becomes necessary to add enriched materials. Examples are blood agar and chocolate agar. Commonly used enriched substances are:

1. Blood (5-10%)
2. Serum (10%)
3. Ascitic fluid (10%)
4. Glucose (1-2%)
5. Plasma (5-10%)

Selective Media: In order to have the best possible chance of isolating a particular type of organism it is important to use a medium that favours its growth and also discourages the growth of unwanted organisms, such a medium is selective. Examples are:

1. BSA (Bismuth Sulphite agar) for salmonella
2. Alkaline peptone water for *Vibrio cholerae*
3. Potassium tellurite agar for *C.diphtheriae.*
4. TCBS for *Vibrio cholerae*
5. DCA for salmonella and shigella

Differential Media: It is a medium to differentiate between the colonies of different organisms. For example, presence of lactose and an indicator in MacConkey agar makes it possible to differentiate between lactose and non-lactose fermenting organisms. Another example is CLED (Cysteine Lactose Electrolyte Deficient) medium.

Enrichment media: Sometimes there is a need to provide enriched environment to some organisms and to inhibit other organisms. These media are usually liquid in nature (broth). The examples are Tetrathionate (TT) and Selenite F broth. In these Salmonella and Shigella species get enriched nutrients, whereas, other intestinal flora like *Escherichia coli and Klebsiella pneumoniae* are inhibited. Sometimes temperature is used for this purpose, cold enrichment is used for *Listeria monocytogenes* and heat enrichment is used for *Legionella* species.

STERILISATION OF MEDIA

Media are sterilised by steaming or by autoclaving. These are discussed in chapter on sterilisation (page 35).

ADJUSTMENT OF PH

pH of the medium is important for a good yield of organisms. It needs to be adjusted before use. *p*H of the medium is estimated by adding an indicator (phenol red) to a measured quantity of the medium (5 ml) and comparing the colour with a set of standards or colour chart. The amount of N/10 HCl or N/10 NaOH to correct the *p*H of 5 ml sample is then determined by titration. Total quantity needed to adjust the reaction of the whole bulk of the medium under preparation is then calculated.

Methods of *p*H measurement

1. pH indicator dyes
2. Electric *p*H meter
3. pH papers

NUTRIENT AGAR

Nutrient agar is a basic culture medium.

Ingredients

Lab-Lemco powder	1.0g
Yeast extract	2.0g
Peptone	5.0g
Sodium chloride	5.0g
Agar	15.0g
Distilled water	1 litre

Preparation: These ingredients are dissolved in a steamer, *p*H is adjusted between 7.2-7.6 and autoclaved at 121°C for 15 min. Medium is then poured in petri dishes.

NUTRIENT BROTH

The formula for the nutrient broth is the same, except that agar is not added in it. Therefore the medium remains in liquid form. It is dispensed in sterile screw capped tubes.

BLOOD AGAR/CHOCOLATE AGAR

Blood agar is an enriched medium, can be made selective by adding Kanamycin or Neomycin for *S.pyogenes*. Heating causes lysis of red cells and medium becomes brown, called chocolate agar. It provides additional nutritional factors to organisms like Haemophilus, Neisseria species and *Streptococcus pneumoniae*. The defibrinated blood is obtained from horse, sheep, goat or rabbit. It should be haemolysis free. The human blood has natural inhibitors, therefore, better be avoided.

Preparation: To make ~70 blood agar plates, melt 1000 ml prepared nutrient agar in a steamer. Cool to 50°C, add 50 ml sterile defibrinated blood, and avoid foaming during mixing. Pour 15 ml into each petri dish. To make chocolate agar, blood agar is heated at 56°C in a steamer, gently mixing every 1-2 min till the colour is changed from red to light brown. This process takes about 6 min. The medium is then poured in plates.

TELLURITE BLOOD AGAR

It is selective medium for the isolation of Corynebacterium diphtheriae.

Preparation: To 200 ml blood agar add 2 ml 3.5% potassium tellurite solution. Mix well and pour 15 ml in each plate. Avoid foaming during mixing and adjust *p*H to 7.4-7.8. This will make about 12 plates, which can be stored at 2-8°C in a sealed plastic bag to avoid loss of moisture for about 10 days.

MACCONKEY AGAR

MacConkey agar is a differential medium distinguish between lactose fermenting and non-lactose fermenting bacteria. It is inhibitory to *Strep pyogenes*, *Strep pneumoniae*, *Strep viridans* and *Pasteurella*. It does not allow growth of Staphylococci if it contains crystal violet.

Ingredients

Peptone	20.0 g
Lactose	10.0g
Bile salt	5.0g
Sodium chloride	5.0g
Neutral red	0.075g
Agar	12.0g
Water up to	1.0 L

Preparation: The ingredients are dissolved in water to make one litre and autoclaved at 121°C for 15 min. The *p*H is adjusted to 7.2-7.6 and is poured in petri dishes. Shelf life is about one month. Store in plastic bags at 2-8°C.

DEOXYCHOLATE CITRATE AGAR (DCA)

It is a heat sensitive, selective and differential medium for Salmonella and Shigella species.

Ingredients

Lab Lemco powder	5.0 g
Peptone	5.0 g
Lactose	10.0 g
Sodium citrate	8.5 g
Sodium thiosulphate	5.4 g
Ferric citrate	1.0 g
Sodium deoxycholate	5.0 g
Neutral red	0.02 g
Agar	12.0 g

Preparation: Dissolve the ingredients in distilled water to make up to 1 litre. Heat in free steam at 100°C for 15 min. *p*H is adjusted to 7.1-7.5 and poured in petri dishes. Plates are packed and kept in plastic bags at 2-8°C up to 6 weeks.

THIOSULPHATE CITRATE BILE SALT AGAR (TCBS)

It is selective and differential medium for Vibrio cholerae and other Vibrio species.

Ingredients

Yeast extract powder	5.0 g
Bacteriological peptone	50.0 g
Sodium thiosulphate	10.0 g
Sodium citrate	10.0 g
Ox-bile	8.0 g
Sucrose	20.0 g
Sodium chloride	10.0 g
Ferric citrate	10.0 g
Bromothymol blue	0.04 g
Thymol blue	0.04 g

Agar	14.0 g
Water up to	1 L

Preparation: Dissolve the ingredients in distilled water to make one litre in a steamer. The final *p*H is adjusted to 8.4-8.8. Plates are packed and kept in plastic bags at 2-8°C up to one month.

SABOURAUD AGAR

It is a routine culture medium for the fungi.

Ingredients

Mycological peptone	10 g
Dextrose	40 g
Agar	15 g
Water up to	1.0 L

Preparation: Dissolve in one litre distilled water in a steamer. Autoclave at 121°C for 15 min. It can be used in petri dishes or slopes in sterile tubes (7-10 ml). *p*H is adjusted to 5.4-5.8. Can be stored in cool, moist places for up to 6 weeks.

DNASE AGAR

It is a medium for detecting DNAse production by *Staph aureus*. The final concentration is 3.9 g per 100 ml of distilled water.

Ingredients

Tryptose	20.0 g
Deoxyribonucleic acid	2.0 g
Sodium chloride	5.0 g
Agar	12.0 g
Water up to	1.0 L

Preparation: The medium is prepared like other media, and poured in petri dishes when cooled to about 50°C. *p*H is adjusted to 7.1-7.5. It can be stored at 2-8°C for 3-4 weeks.

ROBERTSON'S COOKED MEAT MEDIUM (RCM)

It is an enrichment medium, excellent for the rapid growth and maintenance of anaerobic organisms.

Ingredients

Fresh bullock's heart	500 g
Water	500 ml
Sodium hydroxide 1N	1.5 ml
Peptone	2.5 g
Sodium chloride	1.25 g
Concentrated HCl	1 ml

Preparation: Mince the heart and place in boiling alkaline water. Neutralise after 20 min with lactic acid. Drain off the liquid through a muslin filter and while still hot press the minced meat in a cloth and dry partially by spreading it on a cloth or filter paper. 500 ml-filtered liquid is mixed with peptone and sodium chloride. Steam this at 100°C for 20 min, add 1 ml concentrated HCl and filter. The *p*H is adjusted to 8.2, steam at 100°C for 30 min and re-adjust *p*H to 7.8. For the final preparation of the medium, about 2.5 g meat and about 10 ml of the broth is put in one oz bottle. Autoclave at 121°C for 20 min. A tall column of the meat is necessary because anaerobic conditions are present in the depth where there are meat particles.

PEPTONE WATER

This medium is used as basis of carbohydrate fermentation media and to test formation of indole.

Ingredients

Peptone	10g
Sodium chloride	5g
Water up to	1.0 L

Preparation: Dissolve the ingredients in warm water, adjust *p*H to 7.4-7.5, filter and autoclave at 121°C for 15 min.

STANDARD SUGAR SET

Bacteria have the ability to ferment carbohydrates and alcohols. These characteristics are utilised for their identification. Carbohydrates and alcohols comprising standard sugar set are lactose, sucrose, glucose, mannitol, maltose, dulcitol and salicin. In addition, Kosar citrate medium, glucose phosphate medium (MR), peptone water (indole) and a urea slope is included in each set. A phenylalanine agar slope is required for a non-lactose fermenter. Triple sugar iron (TSI) and Krigler iron media are also required. Basic medium is peptone water to which sugars are added. Serum is required for growth of *Neisseriae* and *Corynebacterium.* Therefore, the sugar sets are made in serum-enriched medium (Hiss's serum sugars). Andrade indicator is used in a concentration of 0.005% in these sugar sets. This turns red at *p*H 5.5 and below and remains colourless above *p*H 5.5. A small inverted tube (**Durham tube**) is placed in the glucose tube. It should be completely filled with fluid and should not have any gas bubble. It detects gas production in fermentation process. The gas is seen as an air bubble in this inverted tube. Each tube of sugar set is traditionally identified by colour of cotton wool plug. These are:

Lactose	Red
Sucrose	Blue
Glucose	Green

Mannitol	Mauve
Maltose	Blue and white
Dulcitol	Pink
Salicin	Pink and white

Ingredients

Peptone water	950 ml
Andrade indicator 0.005%	10 ml
10% Sterile solution of sugar	40 ml

Preparation: Indicator and peptone water are mixed and autoclaved at 121°C for 15 min. Sugar solution is sterilised by filtration. Sterile solution of test compound is added on cooling. Dispense in 5 ml quantities in test tubes plugged with corresponding coloured cotton wool. They are stored in a refrigerator.

ALKALINE PEPTONE WATER

It is a transport, enrichment and selective medium for *Vibrio cholerae*. *p*H is adjusted to 8.6 to 9.0, which favours the growth of vibrios and inhibits the growth of other faecal commensals.

Ingredients

Peptone	50 g
Sodium chloride	5 g
Distilled water	500 ml

Preparation: Dissolve ingredients in distilled water and adjust *p*H to 8.6-9.0. The medium is dispensed in 10 ml amounts in screw capped bottles and autoclave at 121°C for 15 min.

TETRATHIONATE BROTH

It is an enrichment medium for typhoid and paratyphoid bacteria. However, it also permits the growth of proteus species.

Ingredients

Solution-I	
Sodium thiosulphate	24.8 g
Sterile water	100 ml
Solution-II	
Potassium iodide	20 g
Iodine	12.7 g
Sterile water	100 ml
Medium	
Calcium carbonate	2.5g
Nutrient broth	78 ml
Solution-I	15 ml
Solution-II	4 ml
Phenol red 0.02 percent in 20 % ethanol	03 ml

Preparation: Calcium carbonate is added to the broth and sterilised by autoclaving at 121°C for 20 min. Thiosulphate, iodine and phenol red solutions are added with aseptically on cooling. The medium can be stored in refrigerator for up to 4 weeks.

MUELLER HINTON AGAR

This medium was originally formulated for isolation of pathogenic *Neisseria* species. However, it is being used for antimicrobial susceptibility testing.

Ingredients

Beef infusion	300 ml
Casein hydrolysate	17.5g
Starch	1.5g
Agar	10g
Distilled Water	1 litre

Preparation: Emulsify starch in cold water, pour into the beef infusion, add casein hydrolysate and agar. Make up to 1 litre with distilled water. Dissolve the constituents by heating gently at 100°C with agitation. Filter if necessary. Adjust *p*H to 7.4. Autoclave at 121°C for 20 min. Pour plates.

LOWENSTEIN-JENSEN GLYCEROL MEDIUM

This medium is used for culture of mycobacteria from different specimens.

Ingredients

1. Mineral salt solution

Potassium dihydrogen phosphate anhydrous	2.4g
Magnesium sulphate	0.24g
Magnesium citrate	0.6g
Asparagines	3.6g
Glycerol	12 ml
Water	600 ml

   Dissolve the ingredients by heating and autoclave at 121°C for 25 min.
2. Malachite green solution: Prepare a 2% solution of malachite green in sterile water. Allow the dye to dissolve by holding at 37°C in the incubator for 1-2h.
3. Egg fluid: Take 20-22 fresh eggs (<4 days old). Wash thoroughly in warm water with a brush and plain alkaline soap, rinse in running water for 30 min. Dry by sprinkling with methylated spirit and burning it off. Crack the eggs with a sterile knife into a sterile beaker and beat them until a uniform mixture is obtained.
4. Complete medium

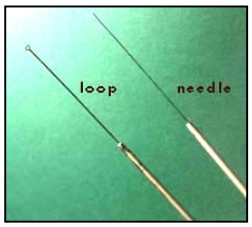
Mineral salt solution	600 ml
Malachite green solution	20 ml
Egg fluid	1 litre

Preparation: Add mineral salt and malachite green to egg fluid. Mix thoroughly, distribute 5 ml amount into 25 ml (McCartney) bottle and screw the cap tightly. Lay the bottles in the inspissator and heat at 80°C for 1h to coagulate and solidify the medium. The slope medium will remain for some months in tightly closed screw-capped bottles.

24. CULTURE TECHNIQUES

INOCULATION OF CULTURE MEDIA

Strict aseptic techniques are to be observed while inoculating a culture medium. It is therefore advisable as far as possible, to carry out inoculation procedures in an inoculation hood (Laminar flow). This will prevent contamination of cultures and specimens. The laboratory staff and the environment will remain free of infection if all the aseptic techniques are applied. The instruments commonly used to inoculate a medium are the platinum or Nichrom loops and needles. The loop consists of a piece of platinum or Nichrom wire, 3 inches long, the free end is bent in the form of a loop. The needle is similar in length but without a loop.The following aseptic techniques are to be observed:



1. Sterilise the wire loops in a flame before and after use. Protect yourself from the dangers of the aerosol. Masks should be used.
2. Flame the mouth of the culture bottles and tubes after removing and before replacing their covers.
3. Decontaminate the table before you start working and after you have finished the day work.
4. Air currents should be reduced to minimum by closing windows and doors and restricting the movement of people in the room.
5. During the inoculation, a culture medium should be uncovered for only a few seconds.
6. Place the lighted Bunsen burner and inoculating instruments to the right of the bench, and cultures and media to the back and the left. (If the operator is right handed).
7. Media to be seeded should be labelled, indicating the inoculum and the date with glass-marking pen, before seeding the plate.
8. Labelling should be on the bottom of the petri dishes and on tubes/bottles rather than on lids or caps.
9. During inoculation the right hand holding the inoculating instrument charged with culture material from the specimen should be moved as little as possible and the left hand should bring the media towards it.

A medium, which has been successfully inoculated, is termed a culture. When only one species of bacterium is grown on the medium it is called a pure culture. If more than one is grown it is called mixed growth. If more than two unidentified colonies are present it is most probably because of contamination. The inoculation of a second medium from a previous culture is termed as subculture.

Seeding a plate

The inoculation of a medium requires practice. The method varies with the medium used. The inoculation of a culture medium in a Petri dish is called plating, looping or seeding and the purpose is to get the isolated colonies of the bacteria from a specimen. This will help to identify the pathogenic organisms by colony characteristics and separating them in pure form for their subculture, biochemical tests, serology if required and doing their sensitivity. In order to economise surface of the culture medium plate, it can be divided into 2-4 parts for plating up to 4 specimens. Plates are dried before inoculation by putting them in incubator at 37°C for about 30-40 min. Lift the bottom of the Petri dish containing medium from its lid with the left hand and hold it round the side with thumb and middle finger. The inoculum is smeared with loop or swab thoroughly over an area at one side of the medium. This area is called as “Well” or **Base**. The loop is re-sterilised and then drawn from the well in two or three parallel lines on to the fresh surface of the medium; this process is repeated, care being taken to sterilise the loop, and cool it on unseeded medium, between each sequence. At each step the inoculum is derived from the most distal part of the immediately preceding strokes. (Figure 24.1 and Figure 24.2). When the inoculum is small or the medium is selective it can be more heavily inoculated. Subculture from liquid media may be distributed with a spreader. This may be bending a piece of glass rod of 3 mm diameter at a right angle in the blowpipe flame, the short limb used for spreading being 2 cm long.

If the medium is in a test tube, this should be

held in the left hand. The platinum loop is held in the right hand and is sterilised in a flame. The plug of the tube is removed by the little finger and the palm of the right hand then the inoculation is carried out and plug is replaced. In inoculating from one tube to another, both tubes should be held between the thumb and the first two fingers of the left hand. The plugs must not be placed on the bench during the inoculation of the tube. Such a practice may result in contamination.

For a slope the loop should be pressed gently but firmly from the surface of the lowest part of the medium and drawn up along the surface to the upper part. In this way the inoculum is thinned as a result of the streaking. The upright media may be used for stab cultures. In a stab culture the charged needle is passed vertically down the centre of the medium. After the plate has been seeded, the bottom of the Petri dish is returned to its lid and the loop is flamed or the glass rod is discarded in a jar containing disinfectant.

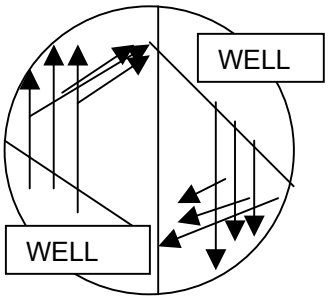


Figure 24.1: Correct Procedure of inoculation of two specimens on one plate.

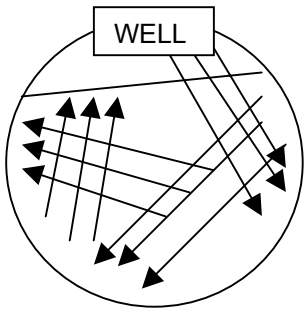


Figure 24.2:Correct Procedure of inoculation of one specimen on one plate.

INCUBATION OF CULTURES

Inoculated media are placed in an incubator at a controlled temperature. Usual temperature is 37°C. Agar plates are incubated in the inverted position, so that the drops of water of condensation forming inside lid do not fall on the surface of the media. The organisms vary in their oxygen requirement and are incubated in the atmosphere according to their requirements. Methods of incubation are:

1. Aerobic method
2. Aerobic with 5-10% CO_2
3. Microaerophilic
4. Anaerobic method

AEROBIC METHOD

In aerobic method, the organisms are incubated in a standard incubator under normal atmospheric conditions at 37°C. However,required temperature may vary in certain cases e.g., 43°C for Campylobacter, 44±0.5°C for faecal *Escherichia coli* (Eijkman test on page 174), 30°C for cultivating leptospira, 32-35°C for oxacillin sensitivity testing for *Staphylococcus aureus*, 22-28°C for fungi and even at 2-4°C for *Listeria monocytogenes*. In order to prevent drying of the medium when prolonged incubation is necessary, as in the cultivation of tubercle bacillus, screw-capped bottles should be used instead of test tubes or Petri dishes.

AEROBIC WITH CO_2

Some organisms, such as *Brucella abortus* (page 144) and capnophilic streptococci require carbon dioxide for their efficient growth. These are termed carboxyphilic bacteria. CO_2 can be provided in incubation atmosphere by two methods.

Candle Jar: Plates to be incubated are placed in a jar. A candle is fixed on top plate and is lighted. Lid of jar is replaced. Candle will consume oxygen in the jar and produce CO_2 and is then extinguished. Jar is now placed in an incubator (see *STREPTOCOCCUS PNEUMONIAE* on page 130 and *NEISSERIA* on page 131).

1. **CO2 Incubators**: Incubators are now available which are connected to gas cylinders containing CO_2. Gas is delivered to inner atmosphere of incubator at a controlled rate to create about 20% concentration. Cultures are placed in these incubators (page 25).

MICROAEROPHILIC METHODS

For the culture of truly microaerophilic species such as *Campylobacter jejuni* and *Actinomyces Israel*, (page 135), an atmosphere of 6-7% O_2 is needed. This can be done by evacuation replacement method with N_2 as the major replacement gas and 5-10% CO_2. The N_2/CO_2 mixture is preferred to H_2/CO_2 mixture, which is potentially explosive. Special gas generating kits

for microaerophilic atmosphere are now available, such as Campy-Pak system (BBL) or Campylobacter Gas generating kit (Oxoid). Alternatively, the gas generating kit for anaerobiosis can also be used without catalyst.

ANAEROBIC METHODS

The strict anaerobic bacteria will not grow in the presence of free oxygen. So in such cases the exclusion of atmospheric oxygen is essential. The following methods may be used for this purpose.

1. **Exclusion of Air from the Medium**: In the case of tall column of medium in a test tube, the deeper layers contain relatively little oxygen if the medium is kept undisturbed. In a liquid medium the dissolved oxygen can be removed by heating the tube and then, allowing the medium to cool undisturbed. The medium is inoculated to the bottom of the tube and the surface of the medium is sealed with sterile Vaseline, or liquid paraffin. The anaerobic organisms will grow in the deeper parts of the medium.
2. **Addition of Reducing Substances**: Sometimes reducing substances are added to medium to make it anaerobic. This ensures the absence of free oxygen and it is a satisfactory way of growing anaerobic organisms in liquid media. It is ineffective in case of surface growths on solid media. Commonly used substances are:
   a. Glucose
   b. Ascorbic acid
   c. Coarsely minced meat particles e.g., in Robertson's Cooked meat medium.
   d. SH Compounds (Sulph-hydryl groups)
   e. Thioglycollic acid e.g., in thioglycollate medium
   f. A piece of soft iron (nail).
3. **Oxygen Free Incubation**: The most satisfactory method of culturing the anaerobic bacteria on solid media is by incubating in a closed jar from which all the oxygen has been removed (page 25).
4. **McIntosh jar**
   a. The usual method is to replace most of the air by hydrogen, and remove the remaining oxygen by making it to combine with hydrogen under the influence of a catalyst. The jar is made of glass, metal or plastic with a well-fitted lid. Asbestos wool impregnated with palladium surrounded by wire gauze is fixed to the under surface of lid. Jar is partly evacuated by a pump, hydrogen is allowed to flow in, and under the influence of the catalyst the residual oxygen is made to react with the hydrogen to form water.

   b. Gas kit: A packet filled with powder is placed in the jar and is made airtight after starting the chemical reaction in the pack. The powder in the kit uses all the free oxygen in the jar in a chemical reaction and thus creates anaerobic atmosphere.

Figure 24.3: Anaerobic cabinet

5. **Anaerobic cabinet**: These cabinets have anaerobic atmosphere with 5-10% CO_2 (Figure 24.3). They have the advantage that all of the processing, including periodic examination of plates and preparation of subcultures, can be done without exposure to O_2 (page 25).

25. BIOCHEMICAL TESTS IN BACTERIAL IDENTIFICATION

There are certain biochemical tests, which are required for identification of enterobacteriaceae. Most of these biochemical tests are usually performed under the common name of "Sugar set". For preparation see under the heading PREPARATION OF CULTURE MEDIA, STANDARD SUGAR SET on page 166.

PEPTONE WATER SUGAR SET

A series of peptone-water sugars; glucose, mannitol, lactose, sucrose, dulcite, and urea can be used for the biochemical differentiation of enterobacteriaceae.

PROCEDURE

Take a sugar set. With a loop touch the colony, and inoculate all the tubes one by one with the same loop. In the end inoculate a blood agar plate with the same loop. This is called the purity plate used later to see that the organism inoculated in the **sugar set** was pure. This **sugar set** is then incubated aerobically at 37°C and the results are read after 18-24 hours.

INTERPRETATION

1. **Carbohydrates**: Pink colour in carbohydrate tubes is taken as positive and no change in colour as Negative. Lactose, sucrose glucose, mannitol, maltose, dulcitol and salicin are seen for production of pink colour as they all have Andrade indicator in them. In glucose tube gas production is also noted in Durham tube (page 166).
2. **Citrate**: Citrate tube is seen for turbidity, which indicates a positive reaction.
3. **Indole**: Take peptone water tube and layer it with a few drops of Ehrlich reagent and shake gently. Look for appearance of a red coloured ring at the upper layer of peptone. This indicates that the organism is indole producer.
4. **Methyl red**: Take glucose phosphate tube and layer it with few drops of methyl red and note the production of red colour at the junction of medium with methyl red (MR positive).
5. **Voges-Proskauer** test: It is performed in same tube if MR test is negative. Add 0.6 ml of 5% α-naphthol, and 0.2 ml 40% KOH. Shake and let the tube stand at room temperature for 15 min. Examine for production of red colour (VP positive).
6. **Urease production**: Urea slope is examined for production of pink colour showing a positive result.
7. **Phenylalanine slope**: Phenylalanine slope is layered with few drops of 3.5% Ferric chloride. If green colour is produced, the result is positive.

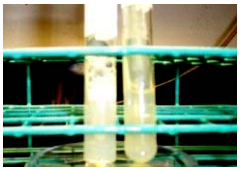
BACTERIAL IDENTIFICATION KITS

Apart from sugar sets made in house, kits based on same principle are available commercially e.g., API, QTS, Enterotube and Systek etc. All reagents for biochemical reactions are present in small wells in dried form. The suspension of the bacteria is added to these wells and reactions are read after 24 hours incubation. Each well has its own code number and the results are read by these codes. A book is provided which translates these codes into name of bacteria. Alternately, the code number can be entered into computer to provide identification of the bacteria. QTS has been developed indigenously by DESTO. Instead of a code system, it works on a flow chart.

CATALASE TEST

Principle: Some organisms contain enzyme catalase, which liberates oxygen from hydrogen peroxide. Test is performed on bacterial growth. It differentiates Staphylococcus from Streptococcus.

Procedure: A small inoculum of test organism is added to a slide or tube having 3% solution of hydrogen peroxide with a sterile wooden or glass rod.



Interpretation: Gas bubbles indicate positive result. Staphylococcus spp is catalase positive and Streptococcus spp is catalase negative.

Control: Use positive and negative controls alongwith the test organisms.

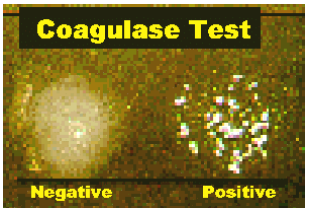
COAGULASE TEST

Principle: Coagulase causes plasma to clot by converting fibrinogen to fibrin. It is done to differentiate *S.aureus* from other staphylococci.

Two types of coagulase are produced by most strains of *Staphylococcus aureus*.

1. **Free coagulase**: It converts fibrinogen to fibrin by activating a coagulase reacting factor present in plasma, detected by tube method.
2. **Bound coagulase (clumping factor)**: It converts fibrinogen directly to fibrin and is detected by slide test.

Procedure (Slide Test): Emulsify a colony in a drop of isotonic saline on each end of slide to make suspension. Add a drop of plasma to one and mix gently. Look for clumping within 5-10 seconds. Other drop serves as negative control to rule out autoagglutination.



Procedure (Tube Test): Half ml 1:6 diluted plasma in isotonic saline is taken in three test tubes, labelled Test, Positive and Negative control. Five drops of broth culture of test organism are added to the tube labelled test, 5 drops of the *Staphylococcus aureus* emulsion to tube labelled positive control, and 5 drops of sterile broth to the tube labelled negative control. Colonies of the *Staph aureus* from blood agar can be used directly for the test. After gentle mixing, these are incubated at 35-37°C and examined for clotting after 1, 3 and 6 hours.

Interpretation: Report "Coagulase positive or negative". In case of negative slide test, tube test must be done before declaring the organism as coagulase negative.

OXIDASE TEST

Principle: The test detects oxidase-producing bacteria and helps in identification of *Vibrio*, *Neisseria* and *Pseudomonas* species.

Reagents: Freshly prepared 10 g/L solution of tetramethyl-*p*-phenylenediaminedihydrochloride (Sigma).

Procedure: Place a piece of filter paper in a clear petri dish and add 2-3 drops of oxidase reagent. Using a sterile glass rod or wooden stick, remove a colony of the test organism from culture plate and streak onto the filter paper. Look for the development of blue purple colour within 5-10 seconds.

Interpretation: Report as oxidase positive if blue purple colour is produced. Test should be controlled by using *Pseudomonas aeruginosa* as positive control and *E.coli* as negative control.

DEOXYRIBONUCLEASE (DNASE) TEST

Principle: Deoxyribonuclease hydrolyses deoxyribonucleic acid (DNA). It is done to identify *S.aureus*.

Procedure: Spot inoculate the test and control organisms on DNA containing culture medium (see DNASE AGAR on page 166). Incubate at 37°C overnight. Flood the surface of plate with diluted hydrochloric acid and tip off the excess. Look for clearing around the colonies after 5 min. Clearing around colonies is produced by DNAse positive strain (page 128).

Control: *Staphylococcus aureus is* positive and *Staphylococcus epidermidis* is negative.

OXIDATION FERMENTATION TEST

This test is used to differentiate organisms that oxidise carbohydrates (aerobic utilisation) such as *Pseudomonas aeruginosa* from those, which ferment carbohydrates (anaerobic utilisation) such as members of enterobacteriaceae family.

Principle: The test organism is inoculated into two tubes of peptone agar medium containing glucose (or other carbohydrate) and the indicator bromothymol blue. The inoculated medium in one tube is sealed with a layer of liquid paraffin to exclude oxygen. Fermentative organisms utilise carbohydrate in both the open and sealed tubes as shown by a change in colour of the medium from green to yellow. Oxidative organisms, however, are able to use the carbohydrate only in open tube. There is no carbohydrate utilisation in the sealed tube (medium remains green). Most bacteria are either carbohydrate oxidiser or slow fermenters, therefore, cultures are usually read after 48 hours but may have to be incubated for 7-14 days.

Reagents

Peptone	2.0 g.
Dipotassium hydrogen phosphate (K_2HPO_4)	0.3 g
Bromothymol Blue (1% aqueous solution)	3 ml
Agar	39 g
Water	11 ml

The *p*H is adjusted to 7.1 before adding the bromothymol blue and the medium is autoclaved at 121°C for 15 min. The carbohydrate to be added is sterilised separately and added to give a final concentration of 1%. The medium is put into tubes to a depth of 4 cm.

Procedure: Duplicate tubes of medium are

inoculated by stabbing with a straight wire. One tube is promptly covered with layer of sterile melted petroleum jelly or liquid paraffin to a depth of 5-10 mm and both are incubated for up to 7-14 days. Fermenting organisms produce acid reaction through out the tube. Oxidising organisms produce an acid reaction only in the open tube; this begins at the surface and gradually extends downwards.

Control: Positive oxidative control is *Pseudomonas aeruginosa,* positive fermentative control is *Escherichia coli*. An un-inoculated tube acts as negative control.

UREASE TEST

Principle: Organisms producing urease split urea to produce ammonia and CO_2. Ammonia changes *p*H of medium to alkaline or is tested by Nessler's reagent. It helps to identify *proteus, H.pylori,* Morganella, Klebsiella and *Y.enterocolitica*.

Reagents

Medium (Christensen's Medium)

Peptone	1g
Sodium chloride	5g
Dipotassium hydrogen phosphate	2g
Phenol red (1 in 500 aqueous solution)	6 ml
Agar	20g
Distilled water	1 litre
Glucose 10 percent sterile solution	10 ml
Urea 20 percent sterile solution	100 ml

Sterilise glucose and urea solution by filtration. Prepare the basal medium, adjust *p*H to 6.8-6.9 and sterilise by autoclaving at 121°C for 30 min. Cool to about 50°C, add glucose and urea, dispense in tubes as deep slopes (The medium may be used as a liquid by omitting the agar).

Procedure: Inoculate heavily the entire slope surface and stab the medium. Incubate at 37°C. Examine after 4 hours and overnight incubation. No tube is reported negative until after 4 days' incubation, although overnight incubation is considered satisfactory. Urease producing organisms change colour of the slope to purple/pink.

Control: *Proteus vulgaris* is positive and *E*.coli is negative.

INDOLE TEST

Principle: This test demonstrates the ability of organisms to breakdown the amino acid tryptophan to indole, detected by Ehrlich or Kovac reagent and helps differentiate enterobacteriaceoe especially *Escherichia coli* which is positive and *Klebsiella pneumoniae* which is indole negative.

Reagents

Kovac reagent

Amyl alcohol	150 ml
p-dimethylaminobenzaldehyde	10 g
Conc. HCl	50 ml

Dissolve aldehyde in alcohol by heating at 50-60°C, cool and slowly add acid. Store in dark, stoppered bottles.

Procedure: Add 0.5 ml Kovac reagent to 3 ml 1-day-old peptone water culture containing tryptophan. A red colour indicates positive test.

Control: *Escherichia coli* is positive and *Enterobacter* sp is negative.

MOTILITY-INDOLE-UREA (MIU)

MIU is a composite medium containing tryptone, phenol red and urea and a paper strip moistened in Kovac's reagent. It is inoculated by a straight wire through the centre of the medium. Non-motile organisms e.g., shigella grow only in the line of the inoculum. Motile organisms (most salmonellae) grow throughout the medium, which becomes turbid. Urease positive organisms (e.g., *proteus spp*) turn the medium red. Indole positive organisms (e.g., *E.coli*) turn the Kovac's strip red (see also TUBE MOTILITY TEST on page 179).

HYDROGEN SULPHIDE (H_2S) PRODUCTION

Principle: H_2S is produced by a large number of bacteria from sulphur containing amino acids. It can be detected by change of colour due to reaction between H_2S and ferrous chloride leading to production of black ferrous sulphide. It helps to differentiate various enterobacteria, and *Brucella* species.

Reagents

Ferrous chloride	10%
Gelatin	120 g
Meat extract	7.5 g
Sodium chloride	3 g
Peptone	25 g
Distilled water to	1 litre

Procedure: Inoculate the medium with straight wire loop to a depth of 1 cm. Incubate at 25-35°C. Inspect daily for change of colour for up to 7 days. Black colour indicates H_2S production.

Control: *Proteus mirabilis* and *Salmonella enteritidis* arepositive, whereas, *E.coli* is negative. Kligler Iron Agar (KIA) is a composite medium containing glucose, lactose, phenol red and ferric citrate. Blackening of the medium indicates H_2S production.

NITRATE REDUCTION TEST

Principle: Some aerobic bacteria reduce nitrates under anaerobic conditions to nitrites, ammonia or nitrogen. Free nitrogen is detected as gas, and nitrites by colour reaction. It is a useful test to differentiate Gram-negative enteric rods and Mycobacterial species.

Reagents

Nitrate agar	
Beef extract	3 g
Peptone	5 g
Potassium nitrate	1 g
Agar	12 g
Distilled water	1000 ml
Sulphanilic acid	
α naphthylamine	

Procedure: Inoculate the medium by streaking the slant and stabbing into butt. Incubate at 35°C for 4 hours. Add 1 drop sulphanilic acid and 1 drop α-naphthylamine to the slant. Red colour indicates positive test.
Control: *E.coli* is positive and *Pseudomonas aeroginosa* are negative.
Plate method of Cook: A strip soaked in 40% potassium nitrate is placed on the centre of a blood agar plate, which is stab inoculated by known positive *Esch.coli* on one side of the strip and test organism on the other. Nitrate positive organisms would produce brown zone as haemoglobin is oxidised to methaemoglobin. It can also be done in broth containing nitrate. After 4 hours, the broth is tested for the reduction of nitrate to nitrite with sulphanilic acid and α-naphthylamine.

EIJKMAN TEST

This is an important test to differentiate faecal coliform organisms (Eijkman positive) from similar bacteria, which are not of faecal origin in water samples. A bottle of MacConkey broth containing a durham tube is inoculated with test organism and incubated in water bath at 44±0.5°C. bacteria that can produce gas from lactose at this temperature give positive reaction indicated by bubble of gas in durham tube within 2 days (see also **Error! Reference source not found.** on page 169 and **Error! Bookmark not defined.**).

ONPG TEST

It demonstrates the presence of enzyme β-galactosidase by utilising *o*-nitrophenol-β-D-galactopyranoside (ONPG). Another enzyme permease is also required to transport lactose into the cell. Lack of permease leads to late lactose fermentation, which can be detected by allowing their β-galactosidase to liberate yellow ortho-nitrophenol from the colourless substrate ONPG.

LECITHINASE

Principle: Certain organisms produce lecithinase, which splits lecithin contained in egg yolk used in the medium (lactose egg yolk milk agar).

Reagents

Egg yolk agar	
Nutrient agar	85 ml
Egg yolk	15 ml

Procedure: Inoculate the organism on media plate with controls. Incubate at 37°C overnight. Look for a zone of opalescence around the colonies indicating lecithinase production (page 134).
Control: *Clostridium perfringens* is positive and control *E.coli* is negative.

AESCULIN HYDROLYSIS

Aesculin is a glycoside incorporated in a nutrient base with a ferric salt. Hydrolysis is indicated by a brown colouration due to reaction of the aglycone (6:7 dihydroxycoumarin) with the iron. Sodium azide is added as preservative.
Principle: Certain organisms hydrolyse aesculin with formation of aglycone, which react with iron to form a brown to black compound. All enterococci, anaerobic cocci, *Streptococcus porcinous, S. uberis, S. suis, S. sanguis, S. bovis, S. equinus, S. mutans, S. salivarius* and *Listeria* spp give positive test. All other streptococci are negative.

Reagents and Media

Aesculin Broth	
Aesculin	1 g
Ferric citrate	0.5 g
Peptone Water	1000 ml

Dissolve aesculin and iron salt in peptone water and sterilise at 115°C for 10 min.
Aesculin Agar: It is aesculin broth gelled by the addition of 2% agar.
Aesculin Cooked meat broth (for anaerobic organisms): 1% aesculin is added to cooked

meat broth before autoclaving. Half ml 1% aqueous ferric ammonium citrate solution is added[1].

Aesculin agar Modified

Aesculin	1 g
Ferric citrate	0.5 g
Blood agar base	40 g
Distilled Water	1000 ml

Dissolve by heating. Cool to 55°C and adjust *p*H to 7.0. Dispense 5 ml screw capped bottles and autoclave at 121°C for 15 min, cool in slopes.

Procedure: Inoculate aesculin broth or agar and incubate at 37°C. Examine daily for 5 days for blackening.

Control: *Enterococcus faecalis* (NCTC 11935) is positive and *Streptococcus agalactiae* (NCTC 11934) is negative.

ARGININE HYDROLYSIS

Arginine is hydrolysed by arginine dihydrolase producing organisms characteristic of certain enterobacteria. Some of streptococci and corynaebacteria are also positive.

Reagents and Media

Nessler's Reagent: Dissolve 5 g potassium iodide in 5 ml fresh distilled water. Add cold saturated mercuric chloride solution until a slight precipitate remains after thorough shaking. Add 40 ml NaOH (9 N). Dilute to 100 ml with distilled water. Allow standing for 24h.

Arginine Broth

Peptone (Tryptone)	5 g
Yeast Extract	5 g
K_2HPO_4	2 g
Glucose	0.5g
Arginine monohydrochloride	3 g
Distilled Water	1000 ml

Dissolve by heating, adjust to *p*H 7.0, boil, filter and sterilise at 115°C for 20 min.

Arginine Agar

Peptone	1.0 g
NaCl	5.0 g
K_2PO_4	0.3 g
Phenol red 1.0% aqueous. Solution	1.0 ml
L (+) arginine Hydrochloride	10.0 g
Agar	3.0 g
D/water	1000 ml

Dissolve in water, adjust *p*H to 7.2, distribute into tubes or screw capped bottles to a depth of about 16 mm (3.5 ml) and sterilise at 121°C for 15 min.

[1] Renew the ferric ammonium citrate solution when its colour changes from green to brown

Procedure-I

Inoculate 5 ml arginine broth. Incubate for 24h at 37°C. Add 0.25 ml Nessler's reagent. A brown colour indicates arginine hydrolysis. (For streptococci add 0.5 ml of culture to 4.5 ml distilled water, shake and add 0.25 ml of Nessler's reagent).

Procedure-II

Stab inoculate into Arginine Agar and pipette on to the surface a layer of sterile liquid paraffin (1 cm depth). Incubate at 37°C. Examine daily for up to 5 days. Positive reaction is indicated by red colour.

Control: *Enterococcus faecalis* (NCTC 8213) is positive, *Streptococcus salivarus* (NCTC 8618 or ATCC 7073) is negative.

PHENYLALANINE DEAMINASE TEST

Principle: Certain members of the family enterobacteriaceae form phenylpyruvic acid from phenylalanine by oxidative deamination. With acidified ammonium sulphate or 10% ferric chloride solution phenylpyruvic acid produces a characteristic green colour. It differentiates proteus and providencia from other enterobacteria and *Y.enterocolitica*.

Reagents

Phenylalanine agar

Yeast extract	3 g
DL Phenylalanine	2 g
Disodium phosphate	1 g
Sodium chloride	5 g
Agar	12 g
Distilled water	1 litre

Dispense into tubes while hot after autoclaving and allow forming slants.

Procedure: Inoculate a slope of phenylalanine agar and incubate at 35-37°C overnight. Add 5 drops of 10% freshly prepared ferric chloride solution to the tube allowing the reagent to run down the slope. Look for green colour on slope within 5 min.

Control: *Proteus vulgaris* is positive and *E.coli* is negative.

LITMUS MILK DECOLOURISATION TEST

Principle: Reduction of litmus milk is indicated by a change in colour of medium from mauve to white or pale yellow. It helps to identify saccharolytic from proteolytic clostridia.

Reagents

1. Solution A (Litmus solution): Grind 80 g litmus granules in 150 ml 40% alcohol and transfer to a flask. Boil for one min and decant to another flask. Add 150 ml 40%

alcohol to boiling flask and boil for one min. Decant to other flask and add HCl drop by drop while shaking till colour turns purple.

2. Solution B (Skimmed milk): Steam milk for 20 min and let stand over night for cream to separate. Siphon the milk to a clean flask.

Litmus Milk Medium: Add 300 ml Solution A and 250 ml Solution B to 500 ml milk. Distribute in 5 ml aliquots to small screw capped bottles. Sterilise by steaming for 20 min at 3 successive days.

Procedure: Heavily inoculate 5 ml sterile litmus milk medium with test organism. Incubate at 35-37°C for up to 4 hours, examining at half hourly intervals for reduction reaction (page 134).

Control: Enterococci and *Cl.perfringens* are positive, *Strep.bovis* and *Strep.viridans* are negative.

CITRATE UTILISATION TEST

Principle: The test is based on utilisation of citrate as only source of carbon, and ammonia the only source of nitrogen. The medium contains sodium citrate, ammonium salt, and bromothymol blue. Growth in the medium is shown by turbidity and a change in colour from light green to blue. It differentiates enterobacteria from other bacteria.

$$\text{Citrate} \xrightarrow{\text{Citrilase-Mg++}} \text{Oxaloacetate} + \text{acetate} \rightarrow \text{pyruvate} + CO_2$$

Reagents

Koser Medium

Sodium chloride	5.0g
Magnesium sulphate	0.2g
Ammonium dihydrogen Phosphate	1g
Sodium citrate	5.0 g
Bromothymol blue (0.2%)	
Distilled water	1 litre

The *p*H is adjusted to 6.8. The medium is dispensed in tubes and sterilised by autoclaving at 121°C for 15 min.

Procedure: Inoculate medium with test organism and Incubate at 35-37°C for 4 days, checking daily for growth and colour change. Avoid contamination of medium with carbon particles from a frequently flamed wire.

Control: *Klebsiella pneumoniae* is positive, *Escherichia coli* is negative. Simon citrate may also be used as an alternative test where blue colour indicates positive result.

GELATIN LIQUEFACTION

Principal: Some organisms produce proteolytic enzymes, which are detected by digestion and liquefaction of gelatin.

$$\text{protein} + \text{water} \xrightarrow{\text{gelatenase}} \text{polypeptides} \xrightarrow{\text{gelatinase}} \text{Aminoacids}$$

Procedure: A stab culture is made in gelatin medium and incubated at 37°C. Tubes are seen daily for liquefaction for 30 days. Remove tubes from the incubator and keep them at 4°C for 30 min before reading the results. Charcoal gelatin discs available commercially; released charcoal particles as indicators of gelatine liquefaction. It is rapid than simple nutrient gelatin method.

Control: *Proteus vulgaris* is positive and *E.coli* is negative.

BILE SOLUBILITY TEST

Principle: Autolytic enzyme of *Streptococcus pneumoniae* cause lysis of broth cultures.

Reagents

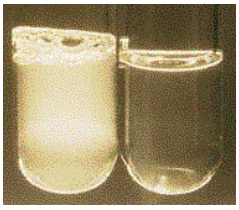
Digest Broth

Meat, finely minced	600 g
Na_2CO_3 anhydrous	8 g
Water	1000 ml
Pancreatic extract (Trypsin extract)	20 ml
Chloroform	20 ml
HCl (conc.)	16 ml

Add alkali and meat to the water, heat to 80°C, stir well and cool. Heat the infusion mixture to 45-50°C, add the pancreatic extract (or trypsin extract) and chloroform, maintain at 45-60°C for 4-6h with frequent stirring. Add acid, boil for 30 min and filter. Adjust *p*H to 8, boil for 30 min and filter. Re-adjust *p*H to 7.6, determine amino acid nitrogen content and dilute to an amino acid nitrogen concentration of 700-750 mg per litre. Sterilise at 115°C for 20 min.

Infusion Broth

Meat, minced	450 g
Water	1000 ml
Peptone	10 g
NaCl	5 g



Allow meat to infuse with the water overnight at 4°C. Skim the fat from the infused mixture, add peptone and salt and boil for 30 min. Filter, adjust *p*H to 7.6 and sterilise at 115°C for 20 min.

Serum Broth

Add 50 ml sterile serum aseptically to 950 ml nutrient broth.

Procedure-I: Inoculate the test organism in 5 ml serum, digest or infusion broth and keep at 37°C for 18 h. Add 0.5 ml of 10% deoxycholate solution. Incubate at 37°C for 15 min. positive test is indicated by loss of turbidity of suspension.

Procedure-II: Suspend centrifuged growth from

broth in PBS[1]. Add 0.5 ml 10% sodium deoxycholate solution. Incubate at 37°C for 15-30 min. Turbidity is lost, if test is positive.
Control: *Streptococcus pneumoniae* ATCC 27336 or NCTC 7465 is positive, *Streptococcus agalactiae* ATCC 13813 or NCTC 8181 and *Streptococcus viridans* is negative.

BILE TOLERANCE

Streptococcus agalactiae, Enterococcus faecalis and other enterococci are resistant to 10-40% bile. Anaerobic bacteria also vary in their ability to grow in the presence of 20% bile. Bile tolerance is helpful in separating the *Bacteroides fragilis* group from other *Bacteroides* spp. and in separating *Fusobacterium mortiferum-varium* from most other clinically significant fusobacteria.

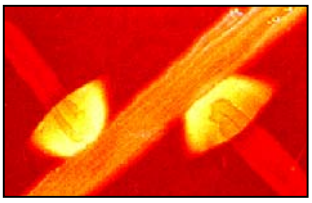
Reagents
Bile Agar: Add 10 or 40 g ox bile[2] (dehydrated) to l L nutrient agar, mix and sterilise at 115°C for 20 min. Cool to ~55°C and distribute.
Thioglycollate Broth

Peptone	15 g
Yeast extract	0.05 g
NaCl	0.05 g
Agar	0.01 g
Thioglycollic acid	0.01 g
Glucose	5 g
Methylene Blue (1% aq solution)	0.02 ml
Water	1 L

Dissolve solids in the water with gentle heat. Add thioglycollic acid, adjust *p*H to 8.5 with 1N NaOH and autoclave[3] at 115°C for 10 min. Adjust *p*H to 7.2, add glucose and dye, mix well and sterilise at 115°C for 10 min.
Oxgall solution: Prepare 40% oxgall solution, sterilise by autoclaving and store at 2 to 8°C.
Procedure I: Inoculate Bile and blood agar with test organism. Incubate at 37°C for 24-48 h. If growth occurs on both plates it is bile tolerant.
Procedure II: Add 0.5 ml 40% oxgall solution in 10 ml warm thioglycollate broth. Inoculate bile and one thioglycollate broth (without bile) with 1 to 2 colonies of test organism. Incubate aerobically for 24-48 h with tight caps. If bile tube reveals growth the organism is bile tolerant.
Control: *Enterococcus faecalis* NCTC 8213 is positive, whereas *Streptococcus dysagalactiae* NCTC 4669 or *Bacteroides melaninogenicus* is negative.

[1] Phosphate buffered saline, pH 7.3.
[2] 10 g ox bile is equivalent to 100 g bile.
[3] To prevent darkening of the medium, screw caps should be loosened during autoclaving

CAMP TEST



Principle: A positive CAMP (Christie, Atkins, Munch, Petersen) test is the production of a clear zone around a colony on sheep or ox blood agar plate affected by staphylococcal α-toxin. Group B streptococci produce a protein like compound called the "CAMP Factor" that is able to act synergistically with α-toxin of *S.aureus* to produce enhanced haemolysis. Similar synergistic haemolysis also occurs with *Corynebacterium ovis* and *Rhodococcus equi*. Phospholipase D, secreted by *Corynebacterium ulcerans,* can prevent this synergistic haemolysis by S.agalactiae and is detected by inhibition of CAMP test. In reverse CAMP test *Clostridium* spp. replaces *Staphylococcus aureus* and a known group A β-haemolytic streptococci exhibits enhanced haemolysis. CAMP Test is positive for *Streptococcus* group B, some of streptococci of groups E, P and U, *Pasteurella haemolytica.* Reverse CAMP Test is positive for *Clostridium perfringens.*
Reagents: Washed sheep erythrocytes are re-suspended in saline to the original volume. CAMP plate is prepared by covering a layer of nutrient base, with another layer containing 10% washed sheep erythrocytes.
Procedure: Inoculate a streak of α-toxin producing *Staphylococcus aureus* (NCTC 7428) in centre of the sheep blood agar plate. Inoculate straight lines of the isolates to be tested at right angles to the staphylococcal streaks, stopping just short of staphylococcal line. Incubate plate at 37°C over night in air or for 6 h in 5-10% CO_2. Observe for an arrowhead shaped zone of enhanced haemolysis at the junction between streptococci and staphylococci.
Procedure (Reverse CAMP test): Use unknown clostridia instead of *staphylococcus* and known *Streptococcus agalactiae.* Enhanced α-haemolysis identifies *Clostridium perfringens*.
Control: *S.agalactiae* ATCC 27956 or NCTC 8181 is positive, whereas *E.faecalis* NCTC 8213 is negative.

POTASSIUM CYANIDE (KCN) MEDIUM

Principle: It is a differential medium. *Klebsiella*, *Citrobacter*, and *Proteus* grow freely, while *Escherichia coli, Salmonella*, and *Shigella* are inhibited.

Reagents

Add 15 ml 5% potassium cyanide solution to 1 L nutrient broth. Dispense in 1 ml quantities in to sterile tubes and stopper quickly. The medium can be stored for 2 weeks at 4°C. The inoculum should be small inoculated with straight wire and the bottles should be completely transparent.

Procedure: Inoculate tubes with a 24-hour broth culture grown at 37°C. Observe daily for 2 days for growth.

Control: *Proteus vulgaris* is positive and *E.coli* is negative.

METHYL RED REACTION

Principle: Methyl red indicator is added to a highly buffered glucose medium to see acid production for differentiation of enterobacteria.

Reagents: Buffered glucose peptone broth.

Methyl red indicator: Dissolve 0.1 g methyl red (*p*H range 4.5-6.0) in 300 ml ethyl alcohol and 200 ml distilled water.

Procedure: Inoculate 5 ml buffered glucose phosphate peptone broth with pure culture of test organism. Incubate at 35°C for 48 hours. Add 5 drops of MR reagent. Red colour indicates acid production and a positive test.

Control: *E.coli* is positive and *Klebsiella pneumoniae* gives negative reaction.

VOGES PROSKAUER (V-P) TEST

The Voges-Proskauer (V-P) Test is done at 37°C, but Hafnia group is positive at temperatures of ≤30°C. The usual incubation period is 24-48 hours; but needs to be extended to 5-10 days for organisms giving a negative reaction. Phosphate may interfere with the reaction, so glucose peptone broth without salt or phosphate may be used. Reaction is positive with *Klebsiella pneumoniae, Enterobacter cloacae, Streptococcus anginosus, Vibrio alginolyticus* and *Staphylococcus aureus*. It is negative with *Escherichia coli*, *Streptococcus pyogenes, and Vibrio parahaemolyticus*. The test can be performed in same tube used for MR, if MR is negative (page 178).

Principle: Some bacteria have the ability to produce acetoin (acetyle methyl carbinol) from glucose fermentation, in an alkaline pH, acetoin is oxidised to diacetyl, which reacts with the guanidine compound in the buffered deoxycholate glucose broth, Creatine is added to prevent false negative results. *p*H is important. Acid *p*H should be avoided. The order of adding reagents needs to be correct.

$$\text{Glucose} \xrightarrow{\text{Fermentation}} \text{acetyl methy carbinol(AMC)}$$

$$\text{AMC} \xrightarrow{+O_2\,(KOH)} \text{Diacetyl}$$

$$\text{Diacetyl} + \text{nitrogenous compound in peptone} \rightarrow \text{Red colour}$$

Reagents

1. α-Naphthol: Dissolve 5 g α-naphthol in ethyl alcohol and make volume to 100 ml. Store in a brown bottle at 4°C.
2. Potassium hydroxide: Weigh 40 g KOH quickly, as it is hygroscopic and will become caustic when moist. Water is added in small amounts, as it will produce heat. Make volume to 100 ml. Store in the refrigerator in a polyethylene bottle.
3. Creatine: Dissolve 1 g in 100 ml HCl (0.1 N).
4. Glucose Phosphate medium (as for Methyl Red reaction). For V-P test for *Bacillus* spp., 1% NaCl in Glucose Phosphate medium should be used.
5. Semi solid medium

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
K_2HPO_4	5g
Glucose	5 g
Agar	3 g

   Dissolve ingredients by heating. Dispense in 2.5 ml volumes in bijou bottles and sterilise at 115°C for 10 min.
6. Glucose Agar: Sterilise 10% glucose solution by filtration and add aseptically to 950 ml nutrient agar already sterilise at 121°C for 15 min. Mix and distribute aseptically.

Procedure:

1. Inoculate 2 ml glucose phosphate broth with the suspected organism from pure colony and incubate at 37°C for 48 hrs. Add 6 drops α-Naphthol and 2 drops KOH solution Gently shake the tube after each addition, and slope the tube without tube cover (to increase the area of air liquid interface). Keep at room temperature for 1 h. Examine after 15 min and 1 h for red colour.
2. Inoculate 2 ml Glucose phosphate broth with the suspected organism from pure colony and incubate at 37°C for 48 hrs. Add 6 drops α-Naphthol, 2 drops of Creatine solution and 2 drops KOH solution. Slope the tube without cover, keep at room temperature and examine after one and four hours for eosin pink colour.
3. Stab inoculate and incubate semisolid medium at 37°C for 1-3 days. Place on the surface, add 1 drop creatine solution and 5 drops freshly prepared 3:1 mixture of α-Naphthol and KOH solution. Shake gently to

aerate and read after 1 h. Positive reaction gives a red colour.

4. Inoculate glucose agar medium and incubate for 18-24 h. Harvest growth with sterile distilled water or saline and make a suspension. Take a small test tube and add 1 drop 10 % glucose 1 drop, 1 drop. 0.2% Creatine, 2 drops 0.025 M Phosphate buffer (pH 6.8) and 2 drops of suspension from Glucose agar. Incubate in water bath at 37°C for two hours. Add 3 drops α-Naphthol and 2 drops KOH solution and shake. Keep at room temperature and read result after 10 min. A positive reaction is indicated by a red colour.

Control: *Klebsiella pneumoniae* ATCC 13883 or NCTC 11935 is positive, whereas *Escherichia coli* ATCC 25922 or NCTC 7475 is negative.

MOTILITY OF ORGANISMS

Motility of the organisms is an important characteristic to differentiate organisms having similar biochemical characteristics e.g., *Klebsiella pneumoniae* is non-motile, whereas *Enterobacter cloacae* is motile. (Both have similar biochemical reactions). Similarly *B. anthracis* is non-motile, whereas non-pathogenic *Bacillus* species are motile. It is useful in preliminary identification of *B. anthracis* isolates. Two methods are given: the wet mount and the tube motility test.

WET MOUNT PROCEDURE

Procedure:

1. Make suspension of a colony of test organisms in distilled water on a glass slide. Alternatively, a loop of medium from a fresh broth culture can be used. Put a cover glass on it. Examine under the microscope using the X40 objective. Discard slides in 0.5% hypochlorite solution as it contains live organisms.
2. **Hanging drop method**: Clean a cover slip. Apply Vaseline at its 4 corners. Put a drop of distilled water in the centre and emulsify a colony of test organisms. Put the glass slide gently on it and hold it up side down. See under microscope with, 10X and then 40X objective. Margins of drops are specially seen. Motile organisms are clearly seen moving rapidly in the field. Non-motile organisms show to-and-fro Brownian motion but these don't move in relation to each other.

TUBE MOTILITY TEST

Procedure: Using the sterile inoculating needle, remove some growth from an isolated, suspicious colony of an 18-24 h culture. Inoculate motility agar medium (Peptone water with 0.2% New Zealand agar, semisolid agar) carefully stabbing the needle 3-4 cm into the medium then withdrawing the needle so that a line of inoculum can be seen. Incubate the tube aerobically at 35-37°C for 18-24 h (see also Motility-indole-urea (MIU) on page 173). Tetrazolium salts may be added to motility media to make them easier to read. The salt is colourless, but as the organisms grow, the dye gets incorporated into the bacterial cells, where it is reduced to an insoluble red pigment.

Interpretation: Non-motile organisms, such as *B. anthraci*s, form a single line of growth that does not deviate from the original inoculum stab. Motile organisms form a diffuse growth zone around the inoculum stab.

Control: *Pseudomonas aeruginosa* ATCC 35032 or equivalent is motile and *Acinetobacter* spp. ATCC 49139 or equivalent is non-motile.

Method control: Control strains should be assayed on each day of testing. Resolving an out-of-control result needs checking the purity and identity of the control strains and repeating the test. Tetrasolium salts may be added to motility medium to make them easier to read. The salt is colourless but as the organism grows, the dye is incorporated into the bacterial cells where it is reduced to an insoluble red pigment formazan.

Table 25.1: Identification of bacteria

Bacteria	Morphology	Cultural Characters	Colony Characters	Identification Reactions
Staphylococcus aureus (on page 128)	Gram-positive Cocci in clusters	Aerobes and facultative anaerobes, grow on ordinary media	2-3 mm, golden pigmented	Catalase positive, coagulase positive, DNAse positive
Staphylococcus epidermidis	"	"	"	Catalase positive, coagulase negative, DNAse negative.
Staphylococcus saprophyticus	"	"	2-3 mm, whitish	Catalase positive, Coagulase negative, Novobiocin resistant
Streptococcus spp (on page 129)	Gram-positive cocci in chain	Aerobes and facultative anaerobes	1-5 mm, β-haemolytic, semi transparent	Catalase negative, Bacitracin sensitive, Lancefield group 'A'

Bacteria	Morphology	Cultural Characters	Colony Characters	Identification Reactions
Streptococcus agalactiae	"	Grow on MacConkey and Islam's agar	"	Catalase negative, Bacitracin resistant, Lancefield group 'B', CAMP test positive
Enterococcus faecalis (on page 129)	Gram-positive cocci in angled pairs.	Grow on ordinary media	β- α- or non-haemolytic	Catalase negative, Aesculin positive, Lancefield group 'D'
Streptococcus pneumoniae (on page 130)	Gram-positive diplococci, lanceolate, Capsulated	Aerobes and facultative anaerobes, Grow on serum or blood agar (Chocolate agar).	1 mm flat smooth colonies develop raised rim (draughtsman) α-haemolytic	Catalase negative, Optochin sensitive, Bile solubility positive, Inulin fermentation, Mouse virulence positive
Streptococcus viridans	Gram-positive cocci in chains.	"	α-haemolytic small colonies	Catalase negative, optochin resistant, bile solubility test negative, inulin fermentation negative, mouse virulence negative
Corynebacterium diphtheriae (on page 132)	Gram-positive rods, 3x0.3 µm, obtuse angled pairs or parallel rows (palisading) or Chinese lettering, Pleomorphic, Neisser or Albert stain granules, beaded or barred appearance. Non motile, non-sporing, non capsulated	Grows on blood or serum media, tellurite media inhibit normal flora and differentiae three types, volutin granules more frequent in cultures on Loeffler's slope	Three types on tellurite agar Gravis: daisy head appearance, haemolysis may be present Intermedius: Non haemolytic, small grey lustre-less, uniform Mitis: Greyish black convex, ground glass, glistening surface, periphery lighter (poached egg appearance), haemolytic	Ferments glucose, maltose, galactose and dextrin. Gravis also ferment starch, Glycogen and produce H_2S, pathogenic strains ferment Trehalose, toxigenicity tests e.g., Elek test, and guinea pig inoculation
Mycobacterium tuberculosis (on page 146)	Slender curved rods, 3x 0.3 µm, parallel bundles, non-motile, non-sporing, non-capsulated, acid and alcohol fast (with 20% H_2SO_4)	Strict aerobe, grows on egg yolk media (L-J medium) in 4-6 weeks	Raised dry mamillated whitish, later yellowish, friable, granular	Grows better at 37°C, guinea pig more susceptible than rabbit, niacin test positive
Mycobacterium bovis	"	"	Flat white colonies with smooth, ground glass surface	Rabbit more susceptible than guinea pig, niacin test negative
Mycobacterium leprae (on page 148)	Curved slender bacillus, Rounded club-shaped or pointed ends, Less acid fast (5% H_2SO_4)	Cannot grow on artificial media, grow in footpad of mice or in Armadillos	Does not grow on artificial media	Morphology in smears and biotypes, acid fast staining
Clostridium perfringens (on page 134)	Gram-positive, spore bearing, large rods 3-8x6-1 µm, non-motile, capsulated	Anaerobic, grow on ordinary media	Haemolytic, large opaque convex, with striated border	Saccharolytic, litmus milk stormy clot reaction, phospholipase, positive Neglar plate, lecithinase production, animal pathogenicity
Clostridium tetani (on page 134)	Slender, Gram-positive, rod 2-5x4-5 µm, motile, peritrichous flagella, oval, sub-terminal spores,-drumstick appearance	Strict anaerobe	Non-haemolytic, fine spreading (feathery) colonies	Gelatin is slowly liquefied, litmus milk no coagulation, RCM digestion and blackening of meat
Actinomyces spp (on page 135)	Gram-positive filaments with Gram-negative areas, Acid fast (1% H_2SO_4), branching may be seen.	Anaerobic or microaerophilic, 5% CO_2 helps, growth enhanced by blood, glucose, or serum	Raised, nodular, cream coloured, opaque, adherent, shake culture colonies 10-20 mm beneath surface	Biochemical reactions, saccharolytic.
Listeria monocytogenes	Gram-positive rods, non-sporing, 2-3X 5 µm in acute angled pairs. Motile actively at 25°C, slowly at 37°C.	Aerobic, can grow on ordinary media	β-haemolytic on blood agar	Characteristic "tumbling" motility
Bacillus spp (on page 133)	Gram-positive large spore bearing bacilli, in chains 4-8x1-5 µm	Aerobic and facultative anaerobe, can grow on ordinary media	Greyish, granular, circular, many margins, medusa head appearance	*B anthracis*: glucose, sucrose, maltose produce acid, no gas production, nitrate reduced to nitrite. Animal pathogenicity tests.

Bacteria	Morphology	Cultural Characters	Colony Characters	Identification Reactions
Neisseria spp (on page 130)	Oval Gram-negative diplococci, flattened or concave opposing edges with parallel axis, 0.8 µm	Aerobe, primary culture in 5-10% CO_2, grow better in blood, serum agar, Thayer and Martin, modified New York media.	Colonies are small, greyish, transparent disks 1-2 mm diameter, No haemolysis	Oxidase positive DNAse negative. Identify by agglutination.*N. gonorrhoea* ferments glucose only while *N. meningitidis* ferments glucose and maltose.
Moraxella catarrhalis	"	Grow on blood and chocolate agar	"	No sugar fomented, oxidase positive, DNAse positive.
Haemophilus influenzae (on page 143)	Gram-negative bacilli, pleomorphic, cocco-bacillary forms capsulated, non-motile.	Aerobe, grow on chocolate agar, a source of X and V factors	1-5 mm, transparent smooth and flat, May be opaque and mucoid	Demonstration of satellitism. Growth in presence of X and V factor.
Bordetella pertussis (on page 144)	Gram-negative cocci-bacilli, uniform in size, non-motile, non-sporing, thumb print appearance, capsule may be present	Enriched media required, Bordet-Gengou is used	Whitish, highly refractile, after 2-3 days incubation, resemble bisected pearls	Agglutination with antisera, animal pathogenicity
Escherichia coli (on page 136)	Gram-negative bacilli 2-4x 0.6 µm, non-sporing, motile	Aerobic, facultative anaerobe, grow on simple media, pink lactose fermenting colonies on MacConkey agar	1-3 mm convex, colourless to greyish and translucent, may be haemolytic	Ferment lactose, glucose, maltose and mannitol and produces indole, MR. positive V-P, Citrate, KCN. Urea negative. Immunodiffusion to detect toxigenic strains, agglutination for detection of enteropathogenic strains
Shigella spp (on page 136)	Gram-negative bacilli 2-4x 6 µm, non-motile	Aerobic, facultative anaerobes, grow on simple media, non-lactose fermenting yellow colonies on MacConkey agar	Same as above	Agglutination reactions, *S. dysenteriae, S.flexneri, S.boydii, S.sonnei*. Lactose not fermented except late by *S.sonnei* and *S.dysenteriae* type I. Mannitol fermented by all except *S.dysenteriae, S.sonnei* and *S.flexneri* (serotype 6) are indole negative *S.dysentriae* type-1 is catalase negative MR. positive, V-P. citrate and urea negative
Klebsiella spp (on page 139)	Gram-negative bacilli, short and thick, capsulated, non-motile	Aerobic and facultative anaerobe, grow on simple media, MacConkey agar	Mucoid colonies 1-3 mm diameter, pink on MacConkey	Do not liquefy gelatin, or produce ornithine decarboxylase, Indole, MR negative, Citrate, urea V-P, KCN positive, ferment glucose (with gas), lactose and inositol
Enterobacter spp (on page 140)	Gram-negative bacilli motile	" "	Same as above except not very mucoid	Liquefy gelatin, produce ornithine decarboxylase, urea negative
Serratia spp (on page 140)	" "	" "	-	-
Proteus spp (on page 139)	Gram-negative bacilli motile, non capsulated, swarming on blood agar	Aerobic, facultative anaerobe, grow on ordinary media and MacConkey agar	Fishy smell, swarming, yellow colonies on MacConkey agar	Phenylalanine and urea positive, ferment glucose with gas, *P.mirabilis* citrate, indole negative, *P.vulgaris* citrate, indole positive
Morganella spp (on page 139)	"	"	No swarming, yellow colonies on MacConkey agar	Phenylalanine positive, urea indole positive, citrate negative, ferments glucose
Providencia spp (on page 139)	"	"	No swarming, yellow colonies on MacConkey agar	Phenyl nine, urea, mannitol, indole, citrate positive in *P.rettgeri*, urea, mannitol negative in the rest.
Citrobacter spp	"	"	May be lactose fermenting or lactose non-fermenting	Indole, V-P negative, Citrate, MR positive, lysine decarboxylase KCN, H_2S, positive, ferments glucose with gas, lactose
Salmonella spp (on page 137)	"	Aerobic and facultative anaerobe, grows on simple media. DCA, TTB and XLD as selective media	Yellow colonies on MacConkey and DCA. 1-3 mm, large greyish, low convex, round, entire margin	Gas produced except *S.*typhi, Urease, KCN, V-P, Indole negative, MR. positive' Citrate positive except *S.*typhi and *S.*paratyphi, Glucose, mannitol, arabinose, dulcitol, salicin positive, serology for O and H antigens
Yersinia spp (on page 143)	Gram-negative cocco-bacilli, 1.5x0.7 µm, Bipolar staining in non-motile *Y. pestis*	Grow on ordinary media, MacConkey agar, better at room temperature, (25°C)	1 mm small, circular and opaque	*Y.pestis* sucrose, indole urea negative, *Y.enterocolitica* and *Y.pseudotuberculosos* urea positive

Bacteria	Morphology	Cultural Characters	Colony Characters	Identification Reactions
Pseudomonas aeruginosa (on page 140)	Gram-negative, non sporing, motile by polar flagellum	Strict aerobes, on ordinary media, produce pigment	Large, low convex, rough, oval in line of inoculation, shiny, produce pigments, blue-green (pyocyanin), yellow-green (fluorescin), dark brown (pyorubin), pale colonies on MacConkey agar	Oxidase positive, Indole H_2S, V-P, MR negative, ferments glucose with gas
Vibrio cholerae (on page 141)	Gram-negative, comma shaped bacilli 2x 5 µm, actively motile by polar flagellum	Aerobic, grow on ordinary media, good in alkaline peptone water	Shiny colonies 1-2 mm, bluish in transmitted light, pale on MacConkey and yellow on TCBS agar	Oxidase positive, ferments glucose, mannitol, maltose but not lactose, dulcitol, arabinose, Indole, DNAse positive, *V cholerae*, biotypes Classical and El tor, 139 serotypes, Important O1 and O139
V. parahaemolyticus	"	"	Pale colonies on MacConkey agar, green on TCBS	Oxidase positive. Indole, V-P, urea negative. Decarboxylase and DNAse positive. Glucose and Mannitol fermented, gas may be produced
Aeromonas spp (on page 142)	Gram-negative bacilli motile, non-sporing non-capsulated	Aerobe and facultative anaerobe, grow on ordinary media	Yellow colonies on TCBS, pale on MacConkey agar	Oxidase, catalase decarboxylase, DNAse positive. Glucose and Mannitol fermented. Gas may be produced
Pleisomonas spp (on page 142)	"	" No growth on TCBS agar	Pale colonies on MacConkey agar	Oxidase positive. Glucose positive. DNAse negative, lysine negative
Brucella spp (on page 144)	Gram-negative round or oval coccobacilli, non-motile, non-capsulated, non-sporing	Aerobic, *B. abortus* requires 5-10% CO_2, grow on enriched media	Smooth, transparent small 1 mm colonies, days to appear	Sugar not fermented, differentiated by media containing basic fuchsin and thionin. *B abortus* inhibited by thionin. *B. suis* inhibited by basic fuchsin. *B. melitensis* not affected, agglutination by antisera. Urease positive
Acinetobacter spp (on page 141)	Gram-negative bacilli may be diplococci like *Neisseria*.	Aerobes, grow on simple media	Yellow colonies on MacConkey agar, round low convex, round	Ferments glucose, nitrate, oxidase negative
Bacteroides spp (on page 142)	Gram-negative rods, vary in size and morphology	Anaerobic, some grow better on enriched media, neomycin blood agar, Robertson's Cooked meat medium	Variable, may be tiny translucent or large grey, circular or irregular	Sensitive to metronidazole, may ferment glucose, sucrose, Some produce gas, indole and H_2S. Lipase shows pearly effect. *B. fragillis* resistant to penicillin, produces black pigment.
Mycoplasma spp (on page 152)	1-2 µm, pleomorphic cocci or filaments, cell wall deficient, non motile	Aerobes, grow on enriched media with less agar (PPLO agar), Ureaplasma require urea	Fried egg appearance after several days	Serology used in diagnosis of clinical infections
Rickettsia spp (on page 151)	Pleomorphic, short rods, singly or pairs inside cells, stained blue by Giemsa	Grow in yolk sac of embryonated eggs or cell cultures	-	Detection of rickettsial inclusions in cells and Weil Felix reaction
Chlamydiae (on page 151)	Gram-negative bacilli, intracellular, stain purple with Giemsa	Grow in egg yolk and MacCoy, HELA-229 cell lines	-	Immunofluorescent staining for antigens and antibodies in serum

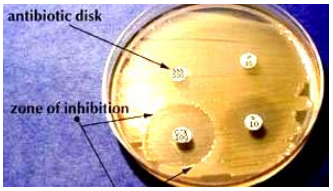
26. ANTIMICROBIAL SENSITIVITY TESTING

Antimicrobial sensitivity testing is one of the most important functions of the clinical Pathological Laboratory. The simplest way of determining the susceptibility of a clinical isolates is to expose it to antibiotics via small paper disks placed on the agar plate. The zone of bacterial growth inhibition around the disk is a degree of efficacy of antibiotic against that organism. Various countries around the world use different methods of performing this test. In UK, the test is a comparative one, in which the susceptibility of the test organism is compared with that of a known, susceptible control strain on the same agar plate (Stoke's method) or on a separate plate (Kirby-Bauer method). The most common method is the standardised test where inhibition zone diameters are compared against standardised zones read from a chart. This is used in many countries; Western Europe uses the ICS (International Collaborative Study) method, France uses the SFM (Societe Francaise de Micobiologie) method, Germany uses the DIN (Deutches Institut fur Normung) method, Scandinavian countries use the SIR (Swedish International Reference) method. However, the method recommended by NCCLS (National Committee for Clinical and Laboratory Standard) in the USA, the Kirby-Bauer method is most widely accepted method in Pakistan and other countries. There are two techniques for putting up sensitivity tests. These are:

1. Disc diffusion technique
2. Agar or broth dilution technique

DISC DIFFUSION TECHNIQUE

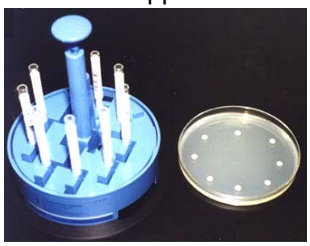
This is used in routine in clinical laboratory. In disc diffusion methods the disks of filter paper soaked in known quantity of antibiotic are placed on plates of appropriate medium inoculated with pure culture of organisms.



Antibiotics diffuse in the surrounding medium thus preventing the growth of organisms in an area where the antibiotic concentration remains sufficient for killing the organisms or preventing their division. A visible clear zone appears the diameter of which, is measured and compared with control organisms. There are two methods of testing antibiotic sensitivity by this technique:

1. Kirby-Bauer method
2. Stokes method

Kirby-Bauer method: Discs are applied on the test strains and control strains in different plates and zones of inhibition of test strains are compared with control strains.



Stokes method: Test and control strains are applied on the same plate in such a way that on one side of the disc is the test strain and on the other side is the control strain. This method is better than the Kirby-Bauer method as the same disc and same medium are used for the test and control strains.

Problems With Disk Diffusion Test

1. The use of correct media is important and diagnostic agars should not be used for susceptibility tests. For example, Muller-Hinton agar for NCCLS method and Iso-sensitest agar for BSAC method are utilised.
2. An inoculum of appropriate density must be used. Too heavy inoculum results in too small zone diameters. Conversely, a light inoculum will produce too large a zone. 0.5 McFarland standard is used to give semi-confluent growth.
3. The antibiotic content of disk is of paramount importance. Too high a concentration, such as may be found in homemade disks, results in false susceptibility reporting. Similarly, incorrect disk storage conditions, especially with β-lactam antibiotics, can adversely affect the potency of the disks and false resistance reporting. The disks for certain β-lactams are kept refrigerated and/or desiccated.
4. Control strains must always be employed, whether the method is comparative or standardised, to ensure the potency of disks.
5. Incubation in an atmosphere containing CO_2 causes a reduction in the *p*H of the medium and can give rise to small inhibition zone when testing macrolides against *Haemophilus influenzae*.
6. Depth of medium: Plates should have a

consistent level depth of 4 mm. Zones of inhibition increases as the depth of agar decreases.

Procedure

1. Select at least four to five well-isolated colonies of the same morphological type from an agar plate culture. Touch the top of each colony with a wire loop and transfer the growth to a sterilised tube containing 4 to 5 ml of a suitable broth medium (e.g., BHI broth).
2. Incubate the broth culture for 2-8 hr at 35-37°C.
3. Adjust the turbidity of broth culture with $BaSO_4$ standard (0.5 unit) by visual comparison, read the tube against a white background with contrasting black lines.
4. Within 15 min after adjusting the turbidity of the inoculum suspension, dip a sterile cotton swab on an applicator into the suspension. Rotate the swab several times pressing firmly on the inside wall of the tube above the fluid level. This will remove excess inoculum from the swab.
5. Inoculate the dry surface of a Mueller-Hinton agar plate by streaking the swab over the entire agar surface. Repeat streaking twice, rotating plate approximately 60° each time.
6. Place the appropriate sensitivity disks on the surface 24 mm apart from centre to centre.
7. Invert the plates and place them at 35-37°C in an incubator within 15 min after applying disks.
8. Examine each plate after 16-18 hours of incubation and measure the diameters of zones of inhibition, including the diameter of the disks.
9. Interpret the sizes by comparing with zones of control strains and/or by referring to the table.

Control Strains

With each batch, the sensitivity of the control strain is also put up. These strains should be sensitive to antibiotics used. These can be obtained from National Collection of Type Cultures (NCTC). The zones of inhibition of test organisms are compared with the zones of inhibition of these organisms. In this way one can check the efficiency of the discs daily. Usual strains are:

- *Staphylococcus aureus*
- *Escherichia coli*
- *Pseudomonas aeruginosa*
- *Clostridium perfringens* (anaerobic)

Sensitivity Media

1. Mueller-Hinton agar is the best of many available media for routine susceptibility test because:
   a. It shows fairly good batch-to-batch reproducibility.
   b. It has low sulphonamide, trimethoprim and tetracycline inhibitors.
   c. It gives satisfactory growth of most pathogens.
   d. A large amount of data, have been collected concerning susceptibility tests performed with this medium.
2. The media containing thymidine or thiamine can reverse the inhibitory effects of sulphonamides and trimethoprim, thus yielding smaller and less distinct zones or even no zone at all. If Mueller-Hinton agar contains thymidine, thymidine phosphorylase or lysed horse blood is added to counteract the effect of thymidine.
3. For some organisms, which do not grow on this agar (e.g., *Streptococcus pyogenes* or *S. pneumoniae*), blood agar or chocolate agar is used for sensitivity testing.

Sensitivity Testing of Bacteria With Special Requirements

The Kirby-Bauer and other diffusion tests have been standardised for rapidly growing pathogens. Larger zones of inhibition will result, if the test is performed with the organisms that have a slow rate of growth, resulting in erroneous findings in the sensitivity testing. Consequently, it is important to give optimal growth conditions to the strains being tested. This may be achieved by using:

Lower incubation temperature: Methicillin resistant *Staphylococcus aureus* (MRSA) may appear sensitive to methicillin when incubated at 37°C, whereas they are resistant at 30°C (or with 5% NaCl added to the medium). This phenomenon is attributed to non-homogeneity of the bacterial population, the resistant part of the population having an optimal growth temperature at 30°C, not being detected at 37°C, because of poor (slower) growth. The following strains may show better growth at 30°C and sensitivity testing at 30°C will give appropriate results:

1. Methicillin resistant staphylococci
2. *Yersinia* spp., *Klebsiella ozaenae*, certain non-fermenter Gram-negative rods
3. *Pseudomonas putida*, *Ps.fluorescens*, some strains of *P.cepacia*, *Aeromonas* spp., and some *Moraxella* spp

Nutritionally supplemented Media: Some strains require supplemented media for growth:

1. Symbiotic streptococci, responsible for bacterial endocarditis require pyridoxine,

thiol or Isovitalex.
2. Strains of enterobacteriaceae forming dwarf colonies on routine media (e.g., thiamine dependent *E. coli, Citrobacter, Klebsiella, Proteus, Salmonella* spp.) require supplement nutrients for larger colony growth. Some strains require CO_2, thiamine, glutamic acid etc., for sensitivity testing.
3. Some strains of *Staphylococcus aureus* form dwarf colonies on routine media and require thiamine and menadione for bettergrowth.
4. Some of the supplemented substances may interfere with the activity of certain antibiotics, e.g., CO_2 affects aminoglycosides, macrolides and tetracyclines, in that a modification of the zone size interpretation should be carried out.

Special interpretation Tables: When testing the sensitivity of slow growing strains or strains with special requirements (*Haemophilus, Neisseria, S. pneumoniae*, anaerobes) special interpretation tables are required.

Sensitivity of *Haemophilus*: The emergence of ampicillin resistant and lately chloramphenicol resistant strains of *H. influenzae* has emphasised the need for routine sensitivity testing of clinical isolates. DST Oxoid Agar, Iso-sensitest agar or Mueller-Hinton agar with low thymidine content, with supplement of 1% haemoglobin (or 5% defibrinated (lysed) horse blood)+1% Iso-vitalex (or supplement B) provide media with no interference with antimicrobials. Chocolate agar can be used if one of above mentioned agar base is used.

1. The bacterial suspension containing 10^5-10^6 CFU/ml is inoculated onto the agar surface with a cotton swab.
2. After drying for 5-15 min, sensitivity disks are placed.
3. The plates are incubated at 35-37°C for 18-24 hours.
4. The diameter of zone of inhibition is measured and sensitivity is determined according to the table.
5. If the isolate is appearing sensitive to ampicillin. It should be tested for β-lactamase production. If not a β-lactamase producer, it should be reported as sensitive, other wise resistant. However, if the test isolate is appearing as resistant on the plate, then there is no need to perform lactamase production test and the isolate should be declared as resistant.

Sensitivity of *Neisseria gonorrhoeae*: The media recommended are DST Agar, Iso-sensitest Agar or Mueller Hinton Agar supplemented with 1% Iso-vitalex and 5% horse blood (1-2% haemoglobin solution). Cysteine-free growth supplement is not required for disk testing. Enriched chocolate agar is not recommended for susceptibility testing of *N.gonorrhoeae*.

Procedure:

1. The direct colony suspension procedure should be used. Using colonies from an overnight chocolate agar culture plate, a suspension equivalent to that of 0.5 McFarland standard is prepared in either Mueller-Hinton broth or 0.9% saline. Within 15 min after adjusting the turbidity of the inoculum suspension, it should be used for plate inoculation.
2. The disk diffusion test procedure as described above for non-fastidious bacteria, should be followed. At the most 9 antimicrobial disks should be placed on the agar surface of a 150 mm agar plate and not more than 4 disks on a 100 mm plate. However, when testing some agents (e.g., quinolones), which produce extremely lager zones, fewer disks may need to be tested per plate.
3. The plates are incubated at 35°C in an atmosphere of 5% CO_2 for 20-24 h.

Zone diameter interpretive criteria: The antimicrobial agents suggested for routine testing of *N.gonorrhoeae* are as follow:

- Cefixime or cefotaxime or cefpodoxime or ceftizoxime or ceftriaxone
- Cefmetazole
- Cefotetan
- Cefoxitin
- Cefuroxime
- Ciprofloxacin or grepafloxacin or ofloxacin
- Penicillin
- Spectinomycin
- Tetracycline

Specific zones diameters interpretive criteria to be used when testing *N. gonorrhoeae* is given in Table 26.1.

Sensitivity Testing of *Streptococcus Pneumoniae* and other *Streptococcus* Spp: The recommended medium for testing *S. pneumoniae* and other streptococci is Mueller-Hinton agar supplemented with 5% defibrinated sheep blood.

Procedure:

1. The direct colony suspension is done. Growth from an overnight (16-8h) sheep blood agar plate is suspended in Mueller-Hinton broth or 0.9% saline to a density equivalent to the turbidity of the 0.5

McFarland standard. It should be used for plate inoculation within 15 min.
2. The disk diffusion procedure steps described above for non-fastidious bacteria, should be followed except that not more than 9 disks should be placed on a 150-mm agar plate nor more than 4 disks on a 100-mm plate.
3. Plates are incubated at 35°C in an atmosphere of 5% CO_2 for 20-24h before measuring the zones of inhibition.

Zone diameter interpretive criteria: The antimicrobial agents suggested for routine testing of *pneumococc*i and other *streptococci* are as follow:

For *Streptococcus pneumoniae*

- Erythromycin
- Oxacillin (for penicillin)
- Trimethoprim/sulphamethoxazole
- Grepafloxacin or Levofloxacin or sparfloxacin or ofloxacin
- Tetracycline
- Vancomycin
- Chloramphenicol
- Rifampicin
- Penicillin, meropenem and cefotaxime or ceftriaxone should be used when zone is ≤19 mm with oxacillin.

Specific zones diameters interpretive criteria to be used when testing *S. pneumoniae* is given in Table 26.1.

For Streptococcus spp. other than *Streptococcus pneumoniae*

- Erythromycin
- Penicillin or Ampicillin
- Chloramphenicol
- Clindamycin
- Vancomycin
- Cefotaxime or ceftriaxone
- Cefipime
- Levofloxacin
- Ofloxacin

Detection of resistant *Staphylococci*

Methicillin/Oxacillin resistance

1. Staphylococci resistant to anti-staphylococcal β-lactamase-stable penicillin (cloxacillin) are labelled as methicillin resistant (MRSA).
2. Oxacillin disk (1 µg) is more likely to detect this resistance than the use of methicillin or cloxacillin disks.
3. The inoculum should be prepared using the direct colony suspension method rather than the inoculum grown method.
4. Test to detect MRSA must be incubated for full 24 hours (rather than 16-18 h) at 33-35°C. (Do not exceed 35°C).
5. Any zone surrounding the oxacillin disk should be inspected carefully using transmitted light for small colonies or a light film of growth within the zone of inhibition.
6. NaCl (2% or 4% w/v; 0.34 or 0.68 mol/L) is added in the Mueller-Hinton agar during its preparation.
7. If the disk diffusion test for MRSA is doubtful then oxacillin-salt agar screening test should be used. In this the Mueller-Hinton agar with 6 µg/ml oxacillin concentration is used as agar diffusion test. The organism is inoculated as spot or streaked on the medium and incubated at 35°C for 24 hours.
8. Agents should be grouped according to the identity of the organisms. The choices of agents for testing will differ from one laboratory to the other depending upon local preference. However, only one member of the group needs to be tested.
    a. Penicillin G is representative of all penicillins when testing staphylococcus.
    b. Methicillin or oxacillin are representative of all penicillinase resistant penicillins.
    c. Ampicillin is representative of amoxacillin.
    d. Cefcaclor, cefadroxil, cephalexin and cephradine are similar and only one need to be tested.
    e. Tetracycline is representative of all tetracyclines.
    f. Clindamycin is representative of lincomycin.
    g. Colistin is representative of polymyxin B.

Selection of Antibiotic Disks

Up to 7-8 sensitivity disks are applied on a single, 90 mm plate. If more disks are required, extended sensitivity can be put on a separate plate. If first 7-8 antibiotics are found resistant or patient is allergic to all, than further antibiotics can be tested. Before reporting an organism sensitive to a particular antibiotic, intrinsic/natural resistance of that organism to a particular antibiotic must be kept in mind. For example, if *Klebsiella* species is found sensitive to ampicillin or *Proteus* species is found sensitive to Nitrofurantoin on plate, they should be disregarded and reported as resistant. This is because all *Klebsiella* species are genetically resistant to ampicillin and all *Proteus* species are genetically resistant to nitrofurantoin.

Table 26.1: Antimicrobial agents, antimicrobial disk contents and acceptable zone diameter for susceptible and resistance

Antimicrobial Agent	Code	Organisms/media	Content (g)	Zone diameter (mm)	
				Resistant ( )	Sensitive ( )
Amikacin	AN		30	14	17
Ampicillin	AMP		10		
		GNR		11	14
		Staphylococci		28	29
		Enterococci		16	17
		Non enterococci		21	30
		Listeria monocytogenes		19	20
		Haemophilus spp.		18	22
Amoxicillin+Clavulanic acid	AMC		20/10		
		Staphylococci and Haemophilus spp.		19	20
		Others		13	17
Ampicillin+ Sulbactam	SAM		10/10		
		Gram negative bacilli and staphylococci		11	15
		Haemophilus spp.		19	20
Aztreonam	ATM		30		
		Haemophilus spp.		-	26
		Others		<15	22
Carbenicillin	CB		100		
		GNR		19	23
		Pseudomonas		13	17
Cefaclor	CEC		30		
		Haemophilus spp.		16	20
		Others		<14	18
Cefamandole	MA		30		
		Haemophilus spp.		20	24
		Others		14	18
Cefazolin	CZ		30	14	18
Cefixime	CFM		5		
		Haemophilus spp.		-	21
		N.gonorrhoeae		-	31
		Others		15	19
Cefoperazone	CFP		75	15	21
Cefotaxime	CFT		30		
		Haemophilus spp.		-	26
		N.gonorrhoeae		-	31
		Others		14	23
Cefotetan	CTT		30		
		N.gonorrhoeae		19	26
		Others		12	16
Cefoxitin	FOX		30		
		N.gonorrhoeae		23	28
		Others		14	18
Cefpodoxime	CPD		10		
		Haemophilus spp.		21	
		N.gonorrhoeae		29	
		Others		17	21
Cefprozil	CPR		30		
		Haemophilus spp.		14	18
		Others		14	18
Ceftazidime	CAZ		30		
		Haemophilus spp.		-	26
		N.gonorrhoeae		-	31
		Others		14	18
Ceftizoxime	ZOX		30		

Antimicrobial Agent	Code	Organisms/media	Content (g)	Zone diameter (mm)	
				Resistant ( )	Sensitive ( )
		Urinary isolates of *Pseudomonas aeruginosa*		10	20
		Haemophilus spp.		-	26
		Others		14	20
Ceftriaxone	CRO		30		
		Haemophilus spp.		-	26
		N.gonorrhoeae		-	35
		Others		13	21
Cefuroxime	CXM		30		
		Haemophilus spp.		20	24
		N.gonorrhoeae		25	31
		Others		14	21
Cephalothin	CF		30	14	18
Chloramphenicol	C		30		
		Haemophilus spp.		25	29
		Others		12	18
Ciprofloxacin	CIP		5		
		Haemophilus spp.		-	21
		N.gonorrhoeae		-	36
		Others		15	21
Clarithromycin	CLR		15		
		Haemophilus spp.		10	13
		Others		13	18
Clindamycin	CC		2	14	21
Colistin	CL		10	8	11
Doxycycline	D		30	12	16
Enoxacin	ENX		10		
		N.gonorrhoeae		-	32
		Others		14	18
Erythromycin	E		15	13	23
Gentamicin	GM		10	12	15
Imipenem	IPM			10	
		Haemophilus spp.		-	16
		Others		13	16
Lomefloxacin	LOM		10		
		Haemophilus spp.		-	22
		Others		18	22
Methicillin	DP		5		
		Staphylococci		9	14
Minocycline	MI		30	14	19
Nalidixic Acid	NA		30	13	19
Neomycin	N		30	12	17
Netilmicin	NET		30	12	15
Nitrofurantoin	F/M		300	14	17
Norfloxacin	NOR		10	12	17
Novobiocin	NB		30		
		Mueller-Hinton agar		17	22
		Mueller-Hinton agar with Blood		14	17
Ofloxacin	OFX		5		
		Haemophilus spp.		-	16
		N.gonorrhoeae		-	31
		Others		12	16
Oxacillin	OX		1		
		Staphylococci		10	13
		Pneumococci		19	20
		(For penicillin G Susceptibility)			
Penicillin	P		10		
		Staphylococci		28	29
		Enterococci		14	15

Antimicrobial Agent	Code	Organisms/media	Content (g)	Zone diameter (mm)	
				Resistant ( )	Sensitive ( )
		non enterococcal *streptococci*		19	28
		L.monocytogenes		19	20
		N.gonorrhoeae		26	47
Piperacillin	PIP		100		
		Pseudomonas		17	18
		Other Gram-negative organisms		17	21
Tetracycline			30	14	19
Tobramycin			10	12	15
	TMP/SMX		1.25/23.75	10	16
Vancomycin	VAN		30	14	17

For Organisms Isolated From Sites Other Than Urine: (Antimicrobials are listed in order of preference)

STAPHYLOCOCCUS
- Penicillin
- Erythromycin
- Cotrimoxazole
- Chloramphenicol
- Oxacillin
- Doxycycline
- Cephalexin/cephradine
- Gentamicin
- Vancomycin/teicoplanin
- Amikacin
- Clindamycin
- Ciprofloxacin/ofloxacin
- Minocycline
- Augmentin
- Rifampicin
- Fusidic acid
- Levofloxacin
- Moxifloxacin

PSEUDOMONAS
- Gentamicin
- Tobramycin
- Ciprofloxacin/enoxacin/ofloxacin
- Amikacin
- Ceftazidime/cefoperazone
- Piperacillin
- Aztreonam
- Cefipime/cefpirome
- Piperacillin-tazobactam
- Imepenem/meropenem
- Levofloxacin/sparfloxacin
- Moxifloxacin

ENTEROBACTERIACEAE
- Ampicillin
- Cotrimoxazole
- Gentamicin
- Doxycycline
- Cephalexin/cephradine
- Ciprofloxacin/ofloxacin/enoxacin
- Tobramycin
- Amikacin
- Ceftriaxone/cefotaxime
- Piperacillin
- Aztreonam
- Ceftazidime
- Cefipime/cefpirome
- Piperacillin-tazobactam
- Imepenem/meropenem
- Levofloxacin/sparfloxacin
- Moxifloxacin

STREPTOCOCCUS PYOGENES (GP. A)
- Penicillin.
- Erythromycin
- Doxycycline
- Chloramphenicol
- Cephradine/cephadroxil
- Vancomycin/teicoplanin
- Cefotaxime/ceftriaxone
- Rifampicin
- Cefipime/cefpirome
- Levofloxacin/sparfloxacin
- Moxifloxacin

PNEUMOCOCCI AND HAEMOLYTIC STREPTOCOCCI
- Penicillin
- Erythromycin
- Doxycycline
- Cefotaxime/ceftriaxone
- Chloramphenicol
- Vancomycin
- Rifampicin
- Cefipime/cefpirome
- Levofloxacin/sparfloxacin
- Moxifloxacin
- Imepenem/meropenem

HAEMOPHILUS
- Ampicillin
- Chloramphenicol
- Cotrimoxazole
- Erythromycin
- Doxycycline
- Cefuroxime
- Ceftriaxone/cefotaxime
- Cefipime/cefpirome

NEISSERIA MENINGITIDIS
- Penicillin
- Chloramphenicol

Cotrimoxazole
Ceftriaxone/cefotaxime

SALMONELLA (From blood)
Chloramphenicol
Cotrimoxazole
Ampicillin
Ciprofloxacin/ofloxacin/enoxacin
Cefotaxime/ceftriaxone
Cefixime

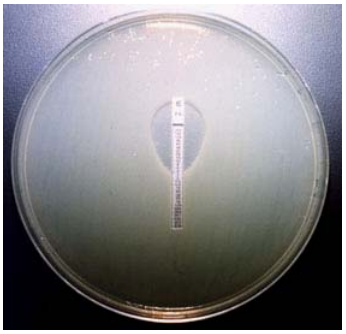
SHIGELLA /DIARRHOEAGENIC *E.coli*
Ampicillin
Cotrimoxazole
Tetracycline
Nalidixic acid
Doxycycline
Chloramphenicol
Ciprofloxacin/ofloxacin/enoxacin

ANAEROBIC ORGANISMS
Penicillin
Metronidazole
Erythromycin
Doxycycline
Chloramphenicol
Amoxycillin-Clavulanic acid
Clindamycin
Cefoxitin/cefotetan
Imepenem/meropenem

Organisms Isolated From Urine

ENTEROBACTERIACEAE (GNR EXCEPT PSEUDOMONAS)
Nitrofurantoin
Ampicillin
Cotrimoxazole
Cephalexin/cephradine
Nalidixic acid
Norfloxacin
Ciprofloxacin/ofloxacin/enoxacin
Gentamicin
Tobramycin
Amikacin
Ceftazidime/ceftriaxone/cefotaxime
Aztreonam
Piperacillin-Tazobactam
Cefipime/cefpirome
Imepenem/meropenem

STAPHYLOCOCCUS
Ampicillin
Cotrimoxazole
Oxacillin (not to be reported)
Doxycycline

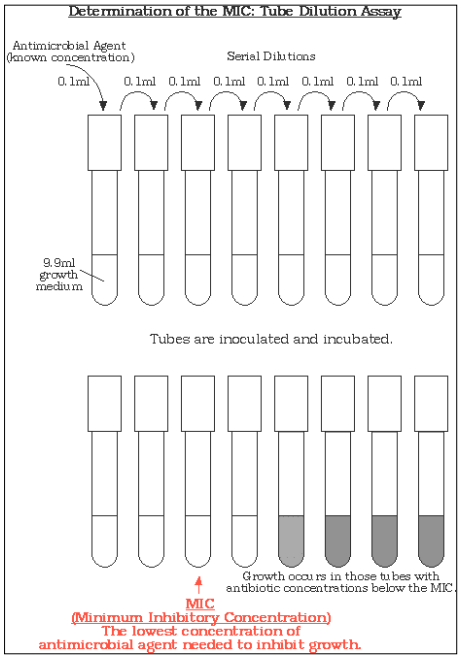


Cephalexin/cephradine
Gentamicin
Pipemedic acid
Norfloxacin
Nitrofurantoin
Amikacin
Ciprofloxacin/ofloxacin
Vancomycin/teicoplanin

PSEUDOMONAS
Norfloxacin
Carbenicillin
Gentamicin
Piperacillin
Ciprofloxacin/ofloxacin/enoxacin
Tobramycin
Amikacin
Ceftazidime/cefoperazone
Piperacillin-tazobactam
Aztreonam
Cefipime/cefopirome
Imepenem/meropenem
Levofloxacin/sparfloxacin
Moxifloxacin

DILUTION TECHNIQUES

These are not used as routine but help to assess the minimum inhibitory concentration (MIC) of a drug. The drug is mixed in solid or liquid medium in different dilutions and the organisms are inoculated on to these media. The lowest dilution showing inhibition of growth is reported as MIC of the drug for that bacterium.



27. BACTERIOLOGICAL EXAMINATION OF WATER

INTRODUCTION

Bacteriological examination of water is routinely conducted to monitor the quality of drinking water. The contaminated water harbours several pathogenic bacteria capable of causing typhoid, dysentery, diarrhoea and cholera. These organisms may be present in water sources contamined by domestic sewage and other pollutants. They are often removed during water purification processes. It is however, desirable to detect their presence in raw as well as in purified drinking water. The detection and estimation of these bacteria is difficult because of presence of very small numbers of organisms and complicated detection techniques, hence the presence of other indicator bacteria such as coliform is routinely monitored for the presence of pathogenic organisms. The coliform group comprises facultative and aerobic gram-negative, non-spore forming rod shaped bacteria that ferment lactose with gas formation in 48 hrs at 37°C. The Coliform bacteria include the genera Escherichia, Enterobacter, & Klebsiella. The *Escherichia coli* are entirely of human origin but their exclusive estimation is difficult and hence the entire coliform are used as indicator. They are reported as an approximate count as 'Most Probable Number' (MPN) by multiple tube fermentation technique (MTF) (Table 27.1).

SAMPLING

The sample should be representative of the bacterial quality; hence extreme care should be taken to avoid contamination. Pre-sterilised (at 121^{o}C for 15 minutes) and paper wrapped glass bottles are used. If the water is known to have residual chlorine, 0.2 ml of 3% sodium thiosulphate solution should be added prior to sterilisation. While sampling in reservoirs, the bottle (still stoppered) is lowered in water (at a depth of 15 to 30 cm). The bottle is held there by the base in one hand, while with the other hand the stopper and cover are removed. These should be retained in hand while the bottle is filled, stopper is then replaced. The filled bottle is finally pulled up. Do not fill the bottle completely, but allow an air space of about 3 cm. While sampling from the taps, the external fittings on the tap are removed and the tap is sterilised by flame on a piece of cotton, soaked in methylated spirit until it is quite hot. The water is then allowed to flow for 2 minutes, the bottle is opened near the tap. Water is collected and the bottle is immediately closed. Avoid any contamination by hand.

TRANSPORT, PRESERVATION AND STORAGE

Samples after collection should be immediately taken to the laboratory for examination. If the processing is not possible within one hour, the samples should be transported in ice. In laboratory, if immediate analysis is not possible, the samples can be preserved at 4°C up to 6 hours, but in no case more than 24 hrs.

MPN OF COLIFORM

Two techniques are available for the estimation of most probable number of coliform in a water sample - the multiple tube fermentation technique (MTF) and the membrane filter (MF) technique. The membrane filter technique involves filtering a known amount of water sample through a membrane filter of optimum pore size. This filter with trapped bacteria is kept on a petri plate with agar medium. These are then inoculated in suitable conditions, the colonies are counted and the bacterial density is calculated per 100 ml of water. The multiple tube fermentation technique is more popular due to its applicability to almost all kinds of waters. The technique involves inoculating the sample and/or multiple dilutions in a suitable liquid medium.

APPARATUS AND MATERIALS

- Test tubes, 25 ml 50 ml
- Durham tubes
- Water bath with a stable 44.5$\pm$0.2°C
- Autoclave

PRINCIPLE

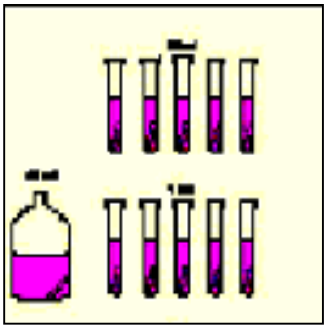
For the waters suspected of having high density, several dilutions are used. As a routine one 50 ml, five 10 ml and five 1 ml volumes of the water sample are inoculated. MacConkey's broth is suitable for this test.

PREPARATION OF MACONKEY'S BROTH (SINGLE STRENGTH MEDIUM)

Bill salts	5 g
Sodium taurocholate) Peptone	20 g
Lactose	10 g
Na Cl	5 g

Bromocresol purple 1% ethanelic solution)	
1% neutral methylred	1 ml
Distilled water	1000 ml
*p*H	7.4 - 7.5

Procedure: Shake all the water samples vigorously immediately before removing sample aliquots to inoculate the series of test tubes. Add sample using sterilised pipettes to the test tubes and mix thoroughly. Place all tubes in an incubator at 35-37°C within 30 minutes. After 48 hrs, examine each tube. Those showing gas in the Durham's vial are recorded as positive (+). Gas in any quantity even a tiny bubble is recorded as (+). The tubes showing positive test are subjected to confirmatory test, as gas production is not the only criterion for a positive test.



Discard all the tubes showing negative test. It is however advisable to examine the tubes first at the completion of 24 hrs. Subject the tubes showing positive test immediately to confirmatory test. Incubate negative tubes to further 24 hrs. The confirmation that the coliform bacilli detected in the presumptive test are *E. coli* is done by Eijkman test (page 174). Tubes showing acid and gas at that temperature contain *E. coli,* the number is read from Table 27.1 *E.coli* can then be confirmed by plating on solid media and testing for indole production and citrate utilisation.

Table 27.1: Most probable number of coliform (McCrady's).

No of tubes giving positive reaction			MPN/ 100 ml	No of tubes giving positive reaction			MPN/ 100 ml
1x50 ml	5x10 ml	5x1 ml		1x50 ml	5x10 ml	5x1 ml	
0	0	0	<1	1	2	0	5
0	0	1	1	1	2	1	7
0	0	2	2	1	2	2	10
0	1	0	1	1	2	3	12
0	1	1	2	1	3	0	8
0	1	2	3	1	3	1	11
0	2	0	2	1	3	2	14
0	2	1	3	1	3	3	18
0	2	2	4	1	3	4	21
0	3	0	3	1	4	0	13
0	3	1	5	1	4	1	17
0	4	0	5	1	4	2	22
1	0	0	1	1	4	3	28
1	0	1	3	1	4	4	35
1	0	2	4	1	4	5	43
1	0	3	6	1	5	0	24
1	1	0	3	1	5	1	35
1	1	1	5	1	5	2	54
1	1	2	7	1	5	3	92
1	1	3	9	1	5	4	161
-	-	-	-	1	5	5	>180

STANDARDS

The classification of drinking water according to bacteriological tests is given in Table 27.2.

Table 27.2: Classification of drinking water

Class	Grade	Presumptive count (per 100 ml)	*E. coli* count (per 100 ml)
I	Excellent	0	0
II	Satisfactory	1-3	0
III	Suspicious	4-10	0
IV	Unsatisfactory	>10	≥0

REPORTING OF RESULT

Mention the presumptive coliform count and *Escherichia coli* count per 100 ml of water. Report it as 'Fit/unfit for human consumption'. Give an advice, if a repeat specimen is required. Make appropriate referral in case of any outbreak of jaundice, cholera, typhoid fever etc.

QUALITY ASSURANCE

1. Due emphasis should be given on proper collection and prompt transportation of the specimen
2. Refrigerate the water specimen for a maximum of 48 hours if not immediately processed.
3. Ensure the reliability of media and instruments.
4. Interpret the results properly

28. MYCOLOGY

INTRODUCTION

The study of fungi is called **Mycology**, and the diseases they cause are called **mycoses**. Fungi exist as unicellular or multicellular organisms, reproducing by the production of spores. The yeasts are unicellular fungi, which reproduce by asexual budding. The cytoplasm of the parent cell is extruded through a hole in the cell wall and a daughter cell is formed, which ultimately breaks away from its parent. This spore is called a **blastospore**, and the typical colony formed is called a yeast colony. Some yeast however, form elongated blastospores or **pseudohyphae**. The multicellular fungi form filaments called **hyphae** on a suitable medium. These structures branch and intertwine forming a meshwork known as **mycelium**. Part of this mycelium is in the medium (**vegetative mycelium**) and part remains on and above the surface (**aerial mycelium**). Hyphae may be **septate** when they have a cross wall in the filaments or **nonseptate**. The reproductive structures formed at the ends of the aerial hyphae are called spores, which can be identified by differences in their appearance. Some of the pathogenic fungi exhibit gross variations in their growth forms according to environmental conditions. Such fungi are called **dimorphic** because they exist as yeast forms in host tissue while growing as molds in the saprophytic state. Most human pathogens are in the taxon Devteriomycetes. They are also called imperfect fungi since they do not reproduce sexually, but produce asexual spores or conidia. There are five types of **asexual** (imperfect) spores, which are of diagnostic value:

1. **Blastospores**: daughter cells formed by budding off from parent cell.
2. **Arthrospores**: formed by segmentation of hyphae into a series of cubical or rounded cells.
3. **Conidia**: These are formed on a specialised hyphae (conidiophore) or borne directly on the side of a hyphae with no apparent conidiophores (Figure 28.1). They may be microconidia (unicellular) or macroconidia (multicellular).
4. **Chlamydospores**: formed by rounding up of a cell with thickening of its wall.
5. **Sporangiospores**: formed within a closed structure called a sporangium, the wall of which ruptures to liberate the mature sporangiospores

Sexual spores: They are rarely found in humans. Basidiospores, ascospores, zygospores are some examples. Yeast cells usually grow as large single cells, rarely forming filaments. They reproduce by asexual process of budding.

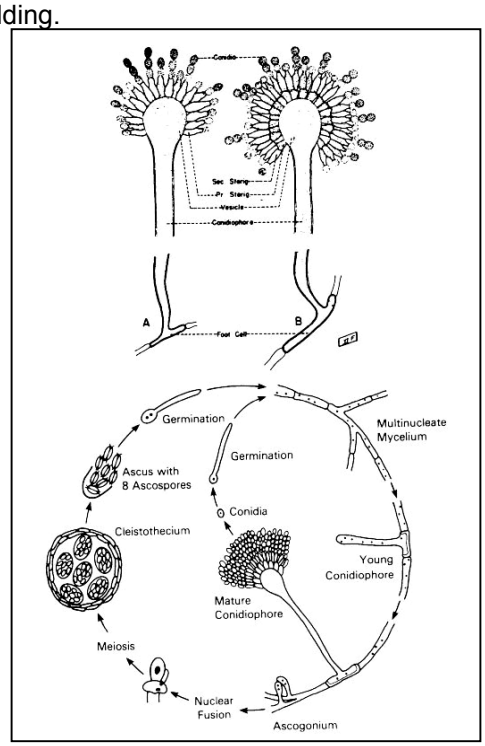


Figure 28.1: Aspergillus spp. Conidial structure and life cycle

FUNGAL INFECTIONS

Fungal infections are classified into three groups depending upon the site of infection and type of fungus.

1. SUPERFICIAL MYCOSES

Infection of the superficial tissue such as skin, hair and nails, is called superficial mycoses. Causative fungi are called **dermatophytes** (Figure 28.2). They belong to following genera.

a. Epidermophyton: *E.floccosum* being the commonest species (Figure 28.3).
b. Microsporum: *M.canis* and *M.gypsium* are the important species (Figure 28.4).
c. Trichophyton: *T.mentagrophytes*, *T.rubrum*, *T.tonsurans* are some of the

important species (Figure 28.5).

d. Pityriasis versicolor – *Malossezia furfur*
e. Black piedra – *Piedraia hortae*
f. Tinea nigra – *Cladosporium werneckii*

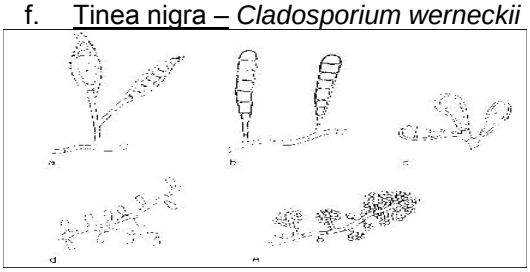


Figure 28.2: Fungal Conidia. a. Macroconidia of Microsporum spp. b. Macroconidia of Trichophyton spp. c. Macroconidia of Epidermophyton spp. d and e. microconidia.

Figure 28.3: Macroconidia of Epidermophyton spp.

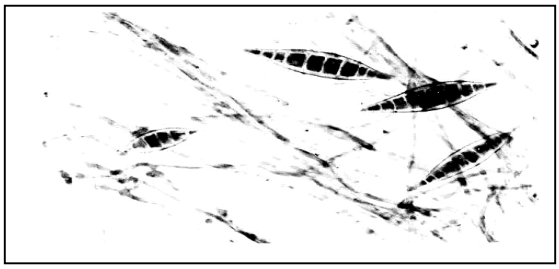


Figure 28.4: Macroconidia of Microsporum spp.

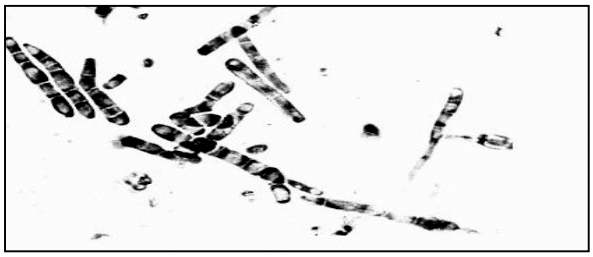
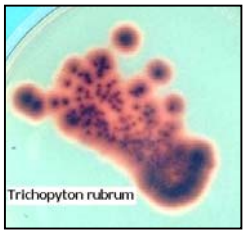
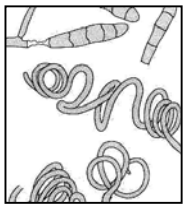


Figure 28.5: Macroconidia of Trichophyton spp.

The colonial morphology on Sabouraud's agar, pigmentation and the characteristic macro and microconidia help to differentiate among them and might help in diagnosing the pathogen up to the species level. *Epidermophyton* spp have rough-walled, philiform macroconidia (Figure 28.3). *Trichophyton* spp have smooth-walled cylindrical macroconidia (Figure 28.5). The diagnostic feature of *T. mentagrophytes* is the production of spiral hyphae. Infections are termed ringworm or tinea. Table 28.1 shows some of the clinical conditions caused by the dermatophytes.





2. SUBCUTANEOUS MYCOSES

These infections are caused by a variety of fungi, found in tropical or sub-tropical regions. Sporotrichosis, chromomycosis and mycetoma are subcutaneous infections caused by fungi like *Sporothrix schenckii*, several species of black moulds. Fungi like *Petriellidium boydii* etc cause mycetoma. Organisms are usually introduced into punctured wound. Infection slowly extends along the lymphatics and eventually localised abscesses are formed. Histologically the lesions are granulomatous. Sporotrichosis is caused by *Sporothrix schenckii,* a dimorphic fungus.

Table 28.1: Fungal Infections with causative fungi and usually involved sites.

Disease	Species	Site
Tinea corporis (Ring worm)	*M.canis, T.mentagrophytes*	Nonhairy and smooth skin
Tinea capitis (Ring worm)	*M.canis, T.tonsurans*	Scalp- hair
Tinea cruris (Jock itch)	*T.rubrum, E.flococosum*	Groin
Tinea pedis (Athlete's foot)	*T.rubrum, T.mentagrophytes, E.floccosum*	Feet (interdigital spaces)
Tinea barbae (barber's itch)	*T.rubrum, T.mentagrophytes*	Bearded facial area
Tinea unguium (Ring worm)	*T.rubrum, T.mentagrophytes, E.floccosum*	Finger nails and toe nails

Mycetoma: It is a subcutaneous fungal infection in which yellow, red or black granules are discharged on surface. These granules are the colonies of causative organisms. Fungi causing mycetoma are:

- *Madurella species*
- *Acremonium species*
- *Aspergillus species*
- *Fusarium species*
- *Petriellidium boyd*ii and many others

Chromomycosis is characterised by the appearance of warty nodules, pathogens include *Phialophora* and *Cladosporium* spp

Actinomyces is the most important having following four species:

- *Actino madura medurae*
- *Actino madura pelletieri*
- *Nocardia brasilliensis*
- *Nocardia asteroides*
- *Streptomyces somaliensis*

3. SYSTEMIC MYCOSES

These fungi are usually found in soil and gain entry into human body by inhalation. Most of these cause respiratory tract infection. From here the fungus can go into systemic circulation and can spread. The fungi included in this group are:

- *Coccidioides immitis*
- *Histoplasma capsulatum*
- *Blastomyces dermatidis*
- *Paracoccidioides brasiliensis*

Cocidioidomycosis: It is caused by *Coccidioides immitis* (Figure 28.6), a soil fungus occurring in the form of arthrospores. When inside human body, it assumes spherical form with multiple small ends containing spores. Serious disseminated form comparable to tuberculosis is observed only in 1% of cases. **Histoplasmosis**: It is caused by *Histoplsma capsulatum*, an intracellular mycosis of the reticuloendothelial system (Figure 28.7). Disseminated infection occurs in infants, elderly or immunosuppressed individuals.

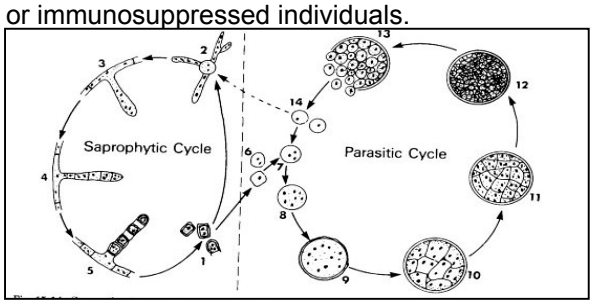


Figure 28.6: Saprophytic and parasitic cycles of Coccidioides immitis.

Paracoccidioidomycosis: It is caused by *Paracoccidioides brasiliensis*, a systemic fungal infection of Latin America.

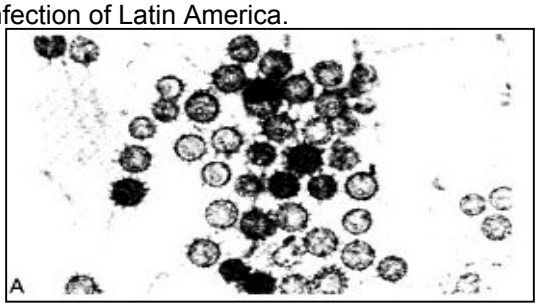


Figure 28.7: Macroconidia of Histoplasma capsulatum .

Blastomycosis: It is caused by *Blastomyces dermatidis* and is a chronic granulomatous disease occurring in American and African continents.

OPPORTUNISTIC MYCOSES

These fungi are present as part of the normal flora and usually do not cause disease in a normal person. These only cause infection when the body defences are compromised. These include:

- Candida species
- *Cryptococcus neoformans*
- Aspergillus
- Zygomyces (Mucormycosis)

Candida albicans: It is the major pathogen, but other candida species may also cause disease. It is a yeast present as part of normal flora of mouth, gastrointestinal tract and vagina. It is Gram-positive and appears as round or oval budding cells of 2-3x4-6 µm or forming pseudohyphae. *Candida albicans* and *C.stellatoidea* give a positive germ tube test (see page 197 for details). Infections are caused in persons on broad-spectrum antibiotics, contraceptive therapy, pregnancy, diabetics and in immunocompromised patients (Figure 28.8). The clinical forms of Candidiasis are:

1. Superficial candidiasis
   a. Cutaneous infection (intertrigo)
   b. Chronic mucocutaneous infection
   c. Onychomycosis (nail infections)
   d. Oropharyngeal infection (thrush)
   e. Vulvovaginitis (thrush)
   f. Keratitis
   g. Conjunctivitis
2. Deep candidiasis
   a. Local inoculation
   b. Oesophagitis
   c. Gastrointestinal candidiasis
   d. Urinary tract infection (fungus ball of the ureter, cystitis, renal abscess, pyelitis) especially in premature neonates.
   e. Peritonitis/intra-abdominal abscess
3. Haematogenous dissemination
   a. Candidaemia
   b. Chronic disseminated candidiasis
   c. Suppurative phlebitis
   d. Endocarditis
   e. Meningitis
   f. Endophthalmitis
   g. Arthritis
   h. Osteomyelitis

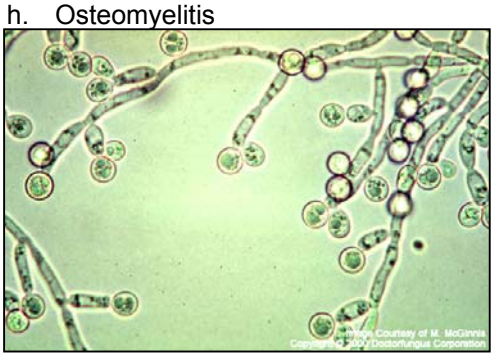


Figure 28.8: Morphogenesis of Candida albicans.

Characteristics of genus candida

1. Colony: carotenoid or melanin pigment absent
2. Cell wall: two layers
3. Shape: variable (globose, elliptical, cylindrical, triangular to lunate)
4. Whole cell hydrolysate: contain no xylose
5. Diazonium blue B colour test: negative
6. Starch like compound: absent
7. Pseudo- or true hyphae: variable
8. Budding: holoblastic, not phialidic
9. Ballistospore: absent
10. Arthroconidia: absent. When invading tissue, it produces pseudohyphae. On Gram film large, Gram-positive, pleomorphic, blastospores are visible. Candida spp grow well on Sabouraud agar or blood agar.

Cryptococcosis: *C.neoformans* is dimorphic yeast, usually associated with opportunistic infection, but may also be a primary pathogen. At ambient temperatures it produces hyphae but at body temperature it is yeast. It gains access through the lung but rapidly disseminates to the CNS to cause cryptococcal meningitis. It grows well on Sabouraud agar or blood agar where it produces mucoid colonies. The mucoid characteristic is imparted by a thick capsule, which can be seen using India ink stain (Figure 28.9). A latex particle agglutination test is also available for rapid diagnosis.

LAB DIAGNOSIS OF CRYPTOCOCCUS NEOFORMANS

1. Direct examination
   - **a.** India ink or nigrosine preparation
   - **b.** Histopathology section (mucicarmine and Masson-Fontana silver)
2. Serological identification
   - **a.** Latex agglutination
   - **b.** Enzyme immunoassay
3. Direct Culture
   - **a.** Niger seed agar medium
   - **b.** Sabouraud agar
   - **c.** Blood agar (Figure 28.9)

Zygomycosis: Pathogens include *mucor* spp, *absidia* and *fusarium* spp. It causes rhinocerebral infection in diabetes mellitus. Pulmonary infection is usually seen in immunocompromised patients on cytotoxic drugs.

Aspergillosis: *A.fumigatus* and *A.niger* are the major pathogens. They are opportunistic pathogens. They can colonise pre-existing lung conditions to cause aspergilloma. It can cause severe and fatal infections in bone marrow transplant (BMT) patients. *A.fumigatus* produces smoky green colonies with a velvety texture.

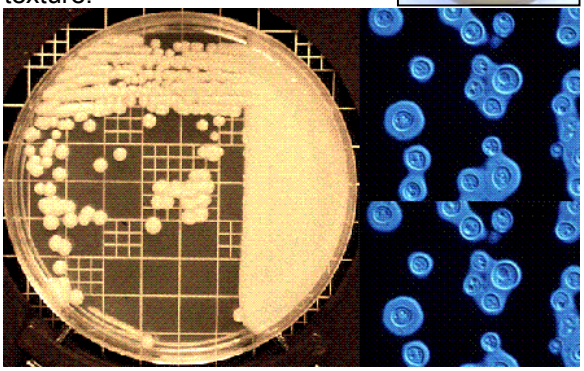


Figure 28.9: Cryptococcus neoformans showing mucoid colonies and thick capsule

LABORATORY DIAGNOSIS OF FUNGAL INFECTIONS

SPECIMENS COLLECTION

Skin: Scrape the active periphery of the skin lesion with a sterile scalpel blade and collect scrapings on a piece of clean paper. Fold the paper and send it to the laboratory.

Nails: Remove affected nails with nail clippers. Clean debris beneath the nail with a blunt probe. Collect and despatch as for skin.

Hairs: Examine the scalp and other hair-bearing areas under illumination of a Wood's lamp (UV light) for fluorescence. Extract fluorescing hairs (infected with Microsporium) with forceps. If no fluorescence is seen, take lustreless or broken hairs, fold in clean paper and send to the laboratory. A plastic massage brush may be used to obtain hair samples for culture.

Mucosa: Collect exudate and if present, any thrush like membrane with cotton-wool swabs.

Sputum, pus and exudates: These specimens are taken into a sterile universal container and examined without delay.

DIRECT MICROSCOPY

Skin scrapings, nails and hairs: The direct microscopic examination is the best method of diagnosing ringworm. The specimen is first softened and cleaned with 40% DMS/KOH (dimethyl sulphoxide/potassium hydroxide). This will digest the keratin surrounding the fungi so that the morphology of the fungi can be seen. A drop of this solution is placed on a clean glass slide; a small piece of specimen is transferred to it, covered with a cover slip and kept in a moist

chamber at 37°C. Time taken to soften the material will depend on the type of the specimen. Hair will take about 10 min and nails up to 30 min. Gentle heating over a flame will reduce the time required to soften/clean the material. As soon as the specimen is softened, examine it under microscope using x10 and x40 objectives. Look for branching hyphae, arthrospores and distinguish them from artefacts like elastic fibres, strands of cotton. The width and cross-walls are characteristics of pure hyphae. In case of hair infection look for the hyphae and arthrospores and note whether they are on the outside of the hair or within it. If the infection is outside the hair it is called **ectothrix**. When the infection is inside the hair substance it is called **endothrix**.

Mucosa: Examine unstained wet preparation or in lactophenol cotton blue microscopically. Gram stained smears are useful.

Sputum, exudates and body fluids: Examine unstained wet or lactophenol cotton blue preparations microscopically. If necessary (opaque material) mount in DMS/KOH with heat gently. Examine sputum after liquefaction and a mucolytic agent (sputolysin). Centrifuge and examine deposit. Prepare a mount using India ink (or nigrosine) to demonstrate encapsulated yeasts (*Cryptococcus neoformans*). Examine exudates macroscopically for white or coloured granules; crush any present between two slides, stain by Gram and modified acid-fast stains. Examine microscopically. Calcofluor white fluorescent stain can also be used for direct examination of fungi in clinical specimens.

CULTIVATION OF FUNGI

Following media are used for fungus cultures:

1. Sabouraud's dextrose agar
2. Sabouraud chloramphenicol/gentamicin agar
3. Sabouraud chloramphenicol/gentamicin with cyclohexamide (Actidone) agar (for dermatophytes)
4. Trypticase Soya broth (for blood culture)

The general nutritional and cultural requirements of fungi differ from those of bacteria. They generally grow slowly than bacteria. Grow best at low *p*H (5.0-6.0) and can tolerate 50% sucrose. They can, therefore, grow on media that would exclude most bacteria. Sabouraud's agar provides all these conditions. Three plates or tubes are inoculated for dermatophytes; one plain Sabouraud, one without cyclohexamide and third with chloramphenicol/gentamicin. Cyclohexamide makes the medium selective for dermatophytes and inhibits the growth of other saprophytic fungi. The medium is incubated aerobically at 22-28°C for 2 weeks and examined daily.

IDENTIFICATION OF FUNGI

1. Once the growth appears on the culture medium, its colonial morphology, rate of growth, colour and presence of pigmentation in the medium is noted.
2. From the growth take a part with a straight needle or wire loop and emulsify in lactophenol blue on a slide, cover with a cover slip and examine under low and dry high power lens. Biochemical tests for identification are rarely needed.
3. Alternatively, press a small piece of clear vinyl tape, e.g., cellotape, adhesive side down, on to the surface of a colony. Remove, and place the tape on to a drop of lactophenol blue on a slide and examine under microscope.
4. When the microscopic appearance of a culture is atypical and characteristic morphology is not seen, a preparation made by **slide culture** is of value. From a 2 mm deep Sabouraud agar plate cut a 1 cm square and place on a sterile glass slide. Inoculate four edges of the block with the fungus under test. Cover the block with a sterile cover slip that is slightly larger than the size of the agar square and transfer the preparation to a moist chamber containing layers of blotting paper soaked in 20% glycerol water. Incubate and examine microscopically, before spores have developed, remove the cover slip and place aside with adherent culture uppermost. Discard the agar, leaving the adherent culture on the slide and one drop of alcohol to both cover slip and slide. Just prior to complete evaporation add one drop lactophenol blue to each preparation. Place a clean cover slip on the slide and a clean slide on the cover slip. Blot and seal with nail varnish. Examine microscopically.

OTHER METHODS OF IDENTIFICATION OF FUNGI

Germ tube test for Candida albicans: Place 0.5 ml of serum (human or horse serum) in a small test tube. Emulsify a small portion of yeast colony obtained after overnight growth of the specimen on Sabouraud agar. Incubate at 37°C for 2 hours. Place a drop of this serum on a slide, put a cover slip and examine microscopically for germ tube production i.e., cylindrical filaments originating from the yeast

cells (page 195).

Gram stain: It can also be used to identify the fungus e.g., candida and cryptococcus are Gram-positive, while other fungi do not stain. This can also differentiate between fungi or actinomyces (Gram positive) as the causative organisms of mycetoma.

Methenamine silver Stain: This is the traditional staining method for histological sections. It is also used for smears of sputum and bronchial fluid for *Pneumocystis carinii*. Fungi stain dark brown.

Periodic acid-Schiff (PAS): This stain has been used for histopathological smears and sections for identification of fungi in tissues. The fungus appears pink to black in colour.

Capsular stain (India Ink preparation): Yeast colony from an overnight growth or the specimen (CSF) is emulsified in a drop of saline on a slide. It is mixed with a drop of India ink and examined under microscope after putting a cover slip on it for the presence of capsules. *Cryptococcus neoformans* has capsule (page 163).

Hyphal and chlamydoconidia production: *Candida* species (with exception of *C.glabrata*) usually produce abundant hyphae. The arrangement of hyphae and blastospores is characteristic of a particular species. Large, highly refractile, thick-walled chlamydoconidia may be seen terminally or on short lateral branches in *C.albicans* isolates. For such production of hyphae and chlamydoconidia, culture of the isolates on following media is required:

1. Potato dextrose agar
2. Cornmeal agar with Tween 80
3. Rice-Tween 80 agar
4. Czapek Dox agar

Biochemical test: These tests include assimilation of carbohydrates and nitrates. Conventional locally made media or commercially prepared biochemical kits (like API 20C) can be used.

Serological diagnosis: Clinical infection by fungi can be diagnosed by serological tests. This is specially required in patients suspected of invasive fungal infection not diagnosed by histopathology or cultures. Serological tests include direct identification of fungal antigen in clinical samples or antibodies in serum by latex and haemagglutination, counterimmunoelectrophoresis (CIE), immunofluorescence, ELISA and complement fixation. The fungi for which such tests are available include *Candida albicans, Cryptococcus neoformans, Aspergillus* species, *Histoplasma capsulatum, Blastomyces dermatitidis, Coccidioides immitis, paracoccidioides brasiliensis, Sporothrix schenckii, Rhizopus* species, *Rhizomucor* species and fungi involved in mycetoma.

LABORATORY FUNGAL CONTAMINANTS

Growth of contaminant fungi is one of the common and important problems experienced in clinical laboratories. One must be able to differentiate between the pathogen and contaminants. Latter either invades the petri dish from the edge (having originated as airborne spores in the laboratory), or from spores carried passively on the inoculum. There are so many saprophytic moulds, which are potential pathogens; therefore, their presence needs to be interpreted correctly. Correlating culture results with direct microscopy of the specimen can sometimes solve this problem. Following is a description of common laboratory contaminant fungi:

Geomyces pannorus: Colonies are slow growing, heaped and folded, cream or white with scant aerial mycelium. They are very like dermatophytes microconidia but smaller (1-3 µm). The species are variable, but seems to be a soil saprophyte on keratin substrate. It may cause low-grade nail infection (Figure 28.10).

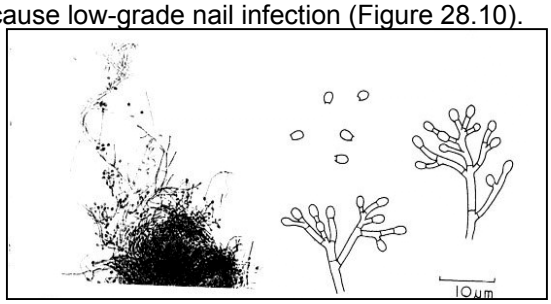


Figure 28.10: Geomyces pannorus.

Chrysosporium species: A large group of dermatophytes having spores resembling microconidia, but larger (5-20 µm). Most form white cream or pale orange colonies, which are flat and suede-like in texture. The group is soil fungus. Human infection is limited to rare, nail infection (Figure 28.11).

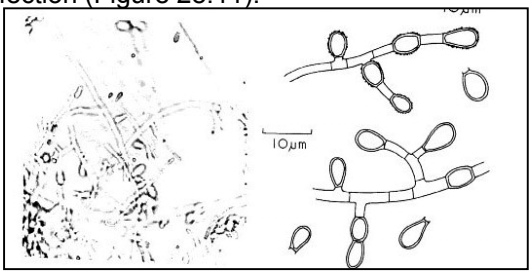


Figure 28.11: Chrysosporium species.

***Trichophyton terrestre* group**: They are

closely related dermatophytes and are saprophytic soil fungus. Colonies are flat, white to cream and densely growing but with a loose superficial mycelium. Unlike pathogenic trichophyton, there is a gradation in spore types from microconidia to macroconidia (Figure 28.12).

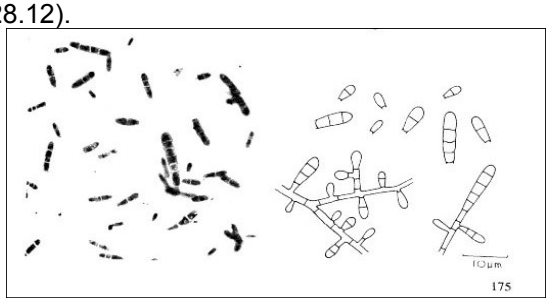


Figure 28.12: Trichophyton terrestre.

Penicillium species: Colonies have shades of green owing to spore pigmentation. Several of the common species are cyclohexamide resistant (Figure 28.13).

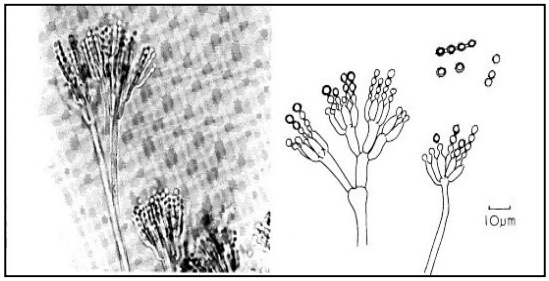


Figure 28.13: Penicillium species.

Trichoderma species: These soil fungi form fast growing, loosely cottony growth, which leaves irregular patches of rich green sporulation adhering to the lid and sides of the petri dish as the mature colony senesces (Figure 28.14).

Paecilomyces species: These differ from species of penicillium in the spore colour; the spores and colony are pale purple or sand brown. They are flat, powdery colonies of medium growth rate.

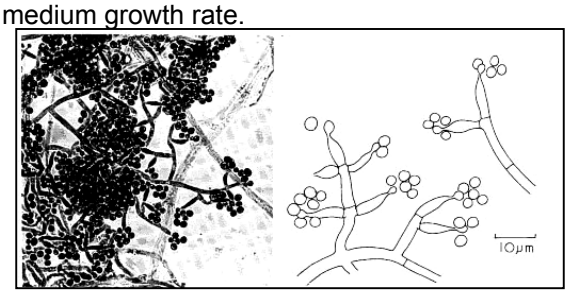


Figure 28.14: Trichoderma species.

Paecilomyces species: They are soil dwellers but occasionally cause deep-seated infections in cold-blooded animals. They have elongated but often single conidiogenous cells. The spores and colony may be purple or sand brown. Colonies are flat and powdery (Figure 28.15).

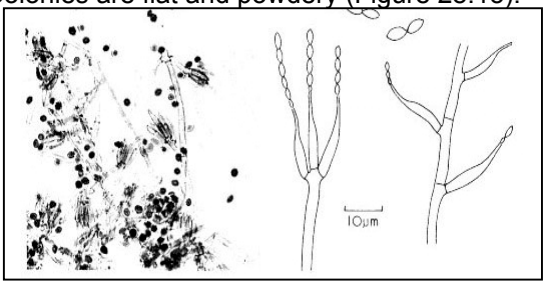


Figure 28.15: Paecilomyces species.

Arthrinium species: They are plant pathogen and have white, fast growing, loose cottony aerial mycelium. The centre of colony becomes black due to black spores. Each spore is shaped like a biconvex lens, densely pigmented except for the rim (Figure 28.16).

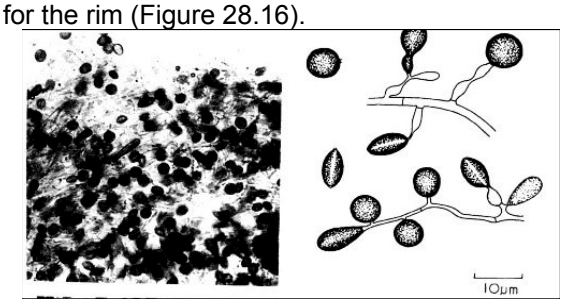


Figure 28.16: Arthrinium species.

Geotrichum candidum: Colonies are flat and grey or white. They have very little mycelium. They resemble yeast colonies but lack budding. All spores are arthroconidia. It is a rare cause of deep mycosis (Figure 28.17).

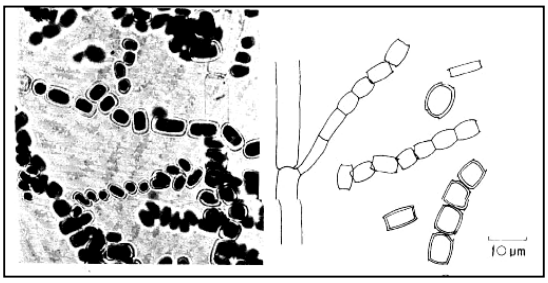


Figure 28.17: Geotrichum candidum.

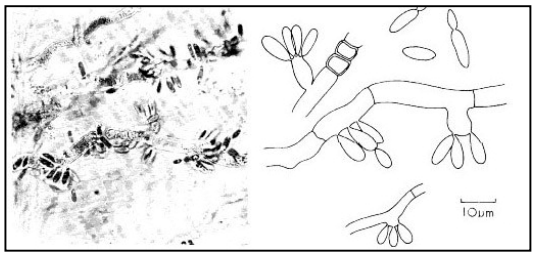


Figure 28.18: Aureobasidium pullulans.

Aureobasidium pullulans: This is plant

parasite and lives on decaying vegetation. Colonies are flat, soft and paste like and wet mucoid with little or no aerial hyphae, white or pale pink at first changing to dark greenish black. The spores are yeast like with secondary budding but mycelial colonies and pigmentation differentiate it. It does not cause infection in man (Figure 28.18).

Beauveria bassiana: This is insect fungus. The colony is pure white, with a dense, aerial tuft. Minute un-pigmented spores are produced at the tip of zigzag shaped filament (Figure 28.19).

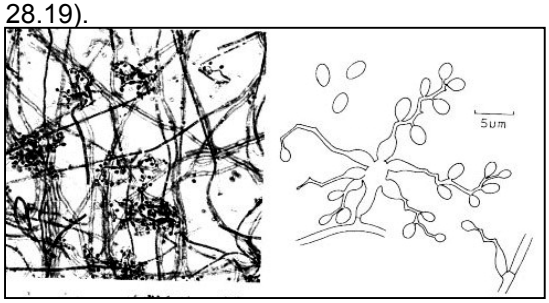


Figure 28.19: Beauveria bassiana.

Acremonium species: They may cause nail infection and eumycetoma in human. The colonies are white, orange or salmon pink, with a low aerial turf, often radially folded. In fluid mounts, usually a single spore remains attached to at least some of the conidiogenous cells (Figure 28.20).

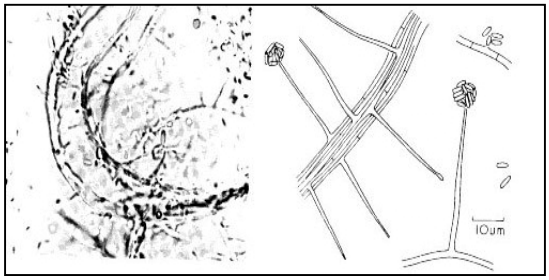


Figure 28.20: Acremonium species.

Curvularia species: Colonies are flat, suede to loosely cottony, dark brown to black. Spores have distinct shape. They cause mycotic keratitis or eumycetoma in man (Figure 28.21).

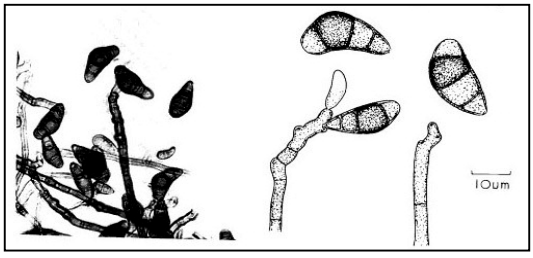


Figure 28.21: Curvularia species.

Alternaria species: They produce cottony white colonies with few spores. The long branching chains of spores readily break up in fluid mounts. They are plant saprophytes. Colonies are flat with thin aerial mycelia on top of dark grey crust made up of spherical to ovoid bodies (pycnidia). Each pycnidium has a definite wall of dark cell and one or more ostioles (Figure 28.22).

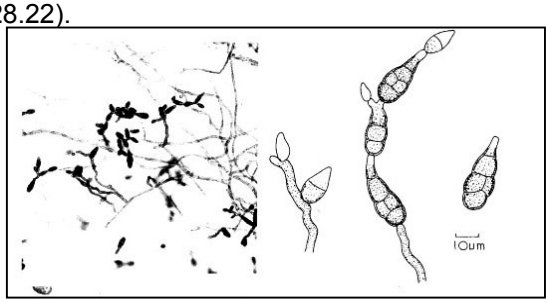


Figure 28.22: Alternaria species.

Ulocladium species: They are plant saprophytes. Colonies are grey to black, powdery to loose cottony. Chains of spores do not develop and spores are more ovoid than the calvate type in Alternaria (Figure 28.23).

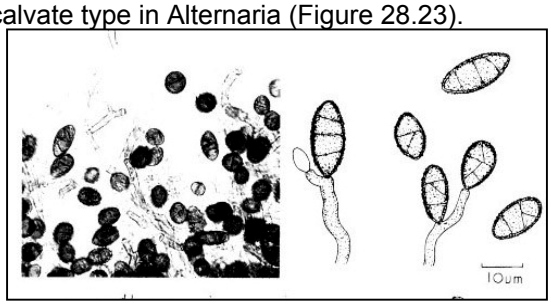


Figure 28.23: Ulocladium species.

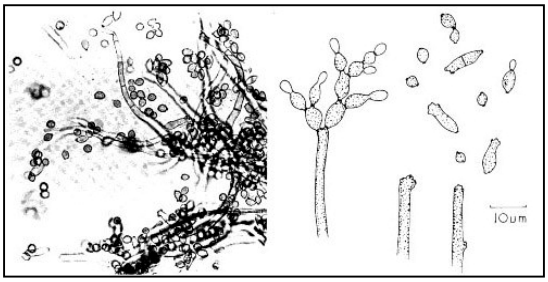


Figure 28.24: Cladosporium species.

Cladosporium species: Colonies are slow growing, often raised or folded, with suede-like surface, olive green to dark grey in colour. Hyphae and spore have brown-pigmented wall on microscopy. A useful feature in identifying the genus is a small dark scar at each end of the spores (Figure 28.24).

Phoma species: They are plant parasites but rarely nail infections and subcutaneous granulomas are present (Figure 28.25).

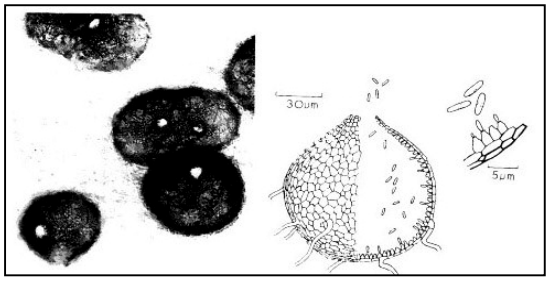


Figure 28.25: Phoma species

Chaetomium species: They are saprophyte on plant material e.g., they may colonise on wallpaper. Colonies are flat, slightly cottony, pale green or greenish brown with minute black bodies (ascomata). Long spines are protruding from the surface of ascomata. Spores are seldom seen, however, dark brown ascospores released from ascomata (Figure 28.26).

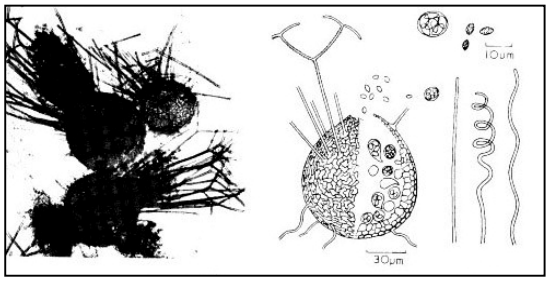


Figure 28.26: Chaetomium species

Myxotrichum species: (Fig-27): They are soil fungi or plant parasites. Colonies are usually white or cream to pale grey-brown, darker in centre, flat or raised, powdery to densely cottony. The large bramble-like masses of interwoven dark hyphae, which form ascomata are readily apparent on low power microscopy (Figure 28.27).

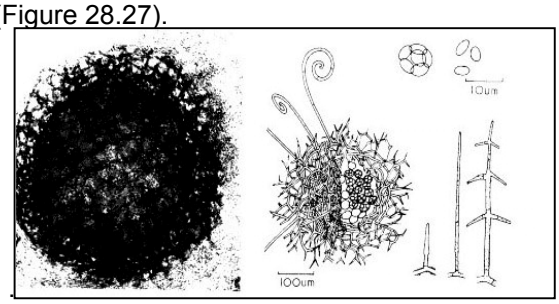


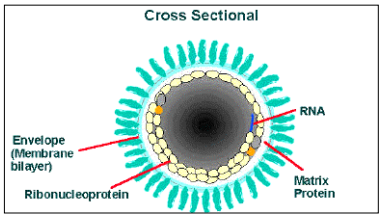
Figure 28.27: Myxotrichum species

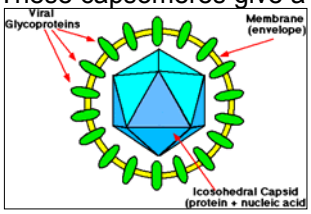
29. VIROLOGY

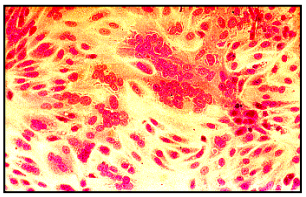
The virology is a branch of pathology, which deals with the diagnosis of viral diseases. The viruses are very small particles and are not visible by light microscopes. The electron microscope is needed to see them. The viruses can pass through filters. These are not having any metabolic activity and are not living organisms. These are taken up by the cells and inside the cell, they invest their genetic material (DNA\RNA). The viral genetic material utilises metabolic machinery of the host cell for the propagation of their genes as well as proteins. The viruses may be plant viruses or animal viruses. The **animal viruses** affect the animals including man. Certain viruses like that of Rabies, Yellow fever, Tick born encephalitis, Lassa fever and Congo Crimean Haemorrhagic Fever (CCHF) are transmitted to human beings from the infected animals. These viral conditions are called as **zoonosis**. Certain viruses are specific to the mankind. The field of medical virology deals strictly with those viruses, which cause disease in the mankind.

BASIC VIRAL CHARACTERISTICS

The viruses vary in size from 18-480 nm in size. They have either RNA or DNA molecule as genetic material. They do not contain cellular organelle like ribosomes or Golgi apparatus. Their **DNA molecule** is either linear or circular in configuration. The DNA is double stranded, except in case of Parvoviruses where it is single stranded with a hairpin like arrangement at one corner. The DNA molecule of Hepatitis B virus is partially (17-51% of the molecule) double stranded. The **RNA genome** is mostly single stranded but that of Reoviruses is double stranded. The RNA molecule is linear as seen in Parainfluenza viruses, Measles virus or Respiratory Syncytial virus. It might be fragmented as in Arenaviruses, Bunyaviruses and Influenzavirus. The RNA genome is not found outside viruses. There are certain RNA viruses, which are unique in their characteristics. These are called Retroviruses. Their RNA genome is first converted to DNA molecule under the action of an enzyme **Reverse Transcriptase**. The resultant DNA is called as proviral DNA molecule and is inserted in the DNA of the host cell. These viruses remain permanently in the body. These are either **oncogenic** (i.e., cause cancer) or cause **acquired immune deficiency syndrome** (AIDS). The viral RNA or DNA genome is covered and protected with ribonucleoprotein. The viruses are covered with number of **capsomeres** that are made up of one or more viral proteins. These capsomeres give a final shape to the virus. The viral structure (capsomeres arranged in an ordered fashion around the other components) is named as **capsid**. The virus may be helical in symmetry, diamond shaped or complicated. Smaller viruses are naked and larger ones are enveloped. Their envelope is made up of cellular membrane taken from the cell last infected, which had been modified by the insertion of viral proteins. That modified cell membrane covers the virus at it exits from the host cell. The naked viruses cannot come out of the infected cell unless the cell is broken (lysed), whereas, the enveloped viruses may bud out of cell without its lysis. They may affect the shape of infected cell that may be rounded up, swollen or fuses with other cells to produce multinucleated giant cells. These cellular changes are distinct in case of different viruses and are called as **cytopathogenic effects (CPE)**. The viruses may be distinguished from each other in a cell culture by the peculiar CPE, neutralisation of CPE, interference to the CPE of other viruses and haemadsorption inhibition. The viral antigen present in the infected cells might also be detected by immunofluorescence based upon the use of specific monoclonal antibodies.







VIRAL PROPAGATION IN THE LABORATORY

The viruses cannot be propagated in an inanimate medium or a culture fluid, as these

are strictly intracellular. They need viable cells for their propagation. Therefore, these may be propagated in the small laboratory animals like newborn mice, fertilised eggs, duck embryo and in cells maintained in culture. The viruses are propagated in cells maintained alive in bottles and tubes (**tissue cultures**) where all the essential requirements for their life are made available in a sterile environment, at body temperature to prevent bacterial infection. The cell cultures are inoculated with the clinical material suspected of containing viruses. These are kept for few days and the CPE is observed.

TYPES OF VIRUSES

The viruses differ from each other as far as their hosts are concerned. There are certain viruses like **Poliovirus**, which can only infect the human beings. Other viruses like **Rabies virus** can infect many types of worm blooded animals. The viruses like that of Yellow Fever and Japanese B

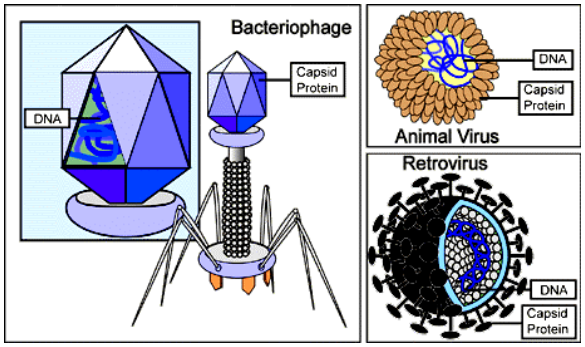


Figure 29.1: Structure of bacteriophage, animal virus and retrovirus

encephalitis are propagated in the mosquitoes as well as warm-blooded animals. They are also called **Arboviruses** (Arthropod borne viruses). The host range is determined by the presence of receptors on the surface of the cells of animal to which a virus may attach and peculiar cellular environment. The receptors are normal constituents of the cell membrane but the viruses utilise them for their own convenience. CD-4 receptor for **Human Immunodeficiency virus (HIV)** is a well known. The Poliovirus affects the intestine and certain neuronal cells. The Mumps virus, on the other hand affects many types of cells like those of heart, pancreas, thyroid, thymus, ovary, testis and brain in addition to the cells of salivary glands. The presence of receptor on its surface, as well as internal environment of the cell determines the potential for the infection of the cell, which should be conducive to viral attachment and propagation.

NOMENCLATURE

The viruses are named differently. Certain viruses are known by the name of the disease they cause. The examples are Rabies virus, Mumps virus, Poliovirus, various Hepatitis viruses (from A to E), Measles virus and Yellow Fever virus. Certain viruses are named after their discoverers like Epstein Barr virus and Dane particles. Certain viruses are given the name of the city or country of their original discovery like Coxsackie A and B viruses, West Nile virus, Japanese B encephalitis and Hazara virus. Certain viruses are named after CPE that they cause, like Respiratory Syncytial virus or Cytomegalovirus. In some cases, more than one characteristic is combined like Enterocyto-pathogenic Human Orphan viruses (Echoviruses). The International Committee of Viral Taxonomy is responsible to assign name to a virus.

CLASSIFICATION

The classification of viruses is complex. These are classified on the basis of type of **genetic material** i.e., DNA or RNA viruses, presence or absence of **envelope**, **shape** and characteristics of their **genome** and the **enzymes** present in the viruses. Important groups are Herpesviruses, Orthomyxoviruses, Paramyxo-viruses, Enteroviruses, Togaviruses, Retroviruses, Papovaviruses, Parvo-viruses and Poxviruses.

CLINICO-EPIDEMIOLOGICAL IMPORTANCE

The viral infections comprise about sixty percent of all human infections. Some of these are universally fatal like rabies and AIDS. Others may be very dreadful like Viral Haemorrhagic Fevers and viral encephalitis that lead to high mortality or permanent damage. Certain viral diseases are of significance in terms of number of chronically affected sufferers and their long-tem complications, like Hepatitis B, C and D. Viruses are incriminated as the causative agents in ~25% of the cancers. Vaccination against several viruses has been extremely effective. The Smallpox was a cause of death in about 10-20% of the mankind. But it has been completely eradicated since 1978 with the help of mass vaccination. Poliovirus is about to be eradicated from most of the world and Measles might be the next target. The viral vaccines make important part of the childhood immunisation campaigns and travel medicine. Hepatitis B vaccine may prevent the high-risk persons from infection, chronic liver disease, and in rare cases from liver cancer.

VIRAL LABORATORY AND WORKERS

The specific/confirmed diagnosis of a particular viral disease is only possible in a laboratory that is equipped with sophisticated equipment and trained staff for this purpose. However, certain initial tests can be carried out in an ordinary laboratory as well. These include screening tests for Hepatitis and HIV, and other tests for determining type of antibody and its titre. Various methods are available for this purpose but the tests based upon **Enzyme Linked Immunosorbant Assay (ELISA)** are the most popular ones. Therefore, a laboratory worker must be well acquainted with the performance of ELISA test and apparatus. He should know the calculation of cut-off point and tabulation of the results. Moreover, he should be familiar with the collection, storage and transportation of specimens. He should know fundamentals of molecular biology. He should be having a thorough understanding of bio-safety, safe handling of the specimens and waste disposal. He should know the use of autoclaves, incinerators and disinfectants.

The specific viral diagnosis should only be undertaken in a referral specialised laboratory that is fully equipped with the storage and maintenance of cell lines, laboratory animals, inverted microscope, fluorescent microscope, electron microscope, molecular biology, serum banking, specialised centrifuges and safety cabinets of different types (see SAFETY CABINETS on page 27). The laboratory should be closed to outsiders. The glassware washing facility must be of top class. The autoclaves should be in perfect functioning order. An intricate system of classification of waste and its proper disposal should exist. The workers must be vaccinated against common viral diseases. They must observe all safety precautions against biohazards and other laboratory hazards.

EMERGENCIES IN VIROLOGY

At times, some procedures in virology have to be done in emergency. In case of renal dialysis, the status of HBsAg is to be known in an hour. In the west, the multiple organ donors are to be tested in emergency for HBsAg, Anti-HIV and Anti-HCV. A quick method is then required. Similarly, in case of health care personnel exposed to needle-stick injury requires the HBsAg test of the source so that the specific immunoglobulin might be administered in time. In case of a vaccinated health care worker, anti-HBs antibody test is to be done to save the prophylactic regimen. The corneal smear for rabies antigen and nasopharyngeal aspirate is dealt at times in emergency.

DIAGNOSTIC PROCEDURES

In a virology department, isolation and identification of disease causing viruses and serological diagnosis of viral diseases is done. The test procedures are complicated ones and the reagents are scarce and expensive. Moreover, patience and professional expertise is required to establish and maintain the optimum conditions for cell culture and molecular techniques. The main duty of peripheral laboratories is to obtain the most suitable and viable clinical material and to transport it without delay to the referral laboratory in a way that the clinical material still remains useful for further processing and testing. Any material not accompanied with properly filled, completed form with date of onset and clinical summary is not acceptable. At times, more than one samples are required. The specimens must be properly labelled and packed in a way that no spillage and breakage of its container occurs during transportation. In case of specialised test and convergence procedures, a prior notice should be given to the referral virus laboratory for making appropriate arrangements.

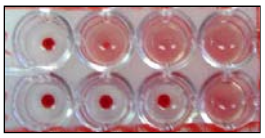
VIRAL SEROLOGY

For making a serological diagnosis, ideally paired specimens of serum are required (see Blood specimen for serology on page 69). One must be obtained as early as possible after the onset of disease. The second specimen should be taken two to three weeks after the onset of the illness: these specimens must be transported in a sterile, well-cleaned plain glass bottle. No antibiotic, additive or preservative is to be added. Septic sera are not acceptable in viral serology. The septic sera may inactivate the complement and the results might not be obtained in case of complement fixation test (CFT). Moreover, such specimen may become sticky and give false positive results in ELISA tests. Aseptic collection, storage of sera at -20°C before transport and quick transport in minimum possible time will prevent sepsis. In following few situations only one serum specimen may

suffice:

1. To establish susceptibility or immunity against some viral disease like Hepatitis A & B, Rabies, Rubella and Poliomyelitis.
2. For Hepatitis B, C virus or HIV (AIDS related virus) serology.
3. To investigate congenitally acquired viral disease in newborns.
4. For estimation of IgM antibodies against certain viral diseases.

The main tests done for sero-diagnosis are CFT (Complement fixation test), HAI (Haem-agglutination inhibition), ELISA (Enzyme linked immunosorbant assay),



Reverse passive haemagglutination (RPHA) or latex agglutination (see also section on PRACTICAL PROCEDURES IN IMMUNOLOGY on page 221 for details). The planning for the most appropriate tests in virology entirely depends upon clinical information. In any case, a brief summary of clinical notes, date of onset and provisional diagnosis must be mentioned. In case many specimens are obtained from the same patient, each specimen must be labelled properly and the date of its collection must be clearly marked. The specimen of serum or CSF meant for a viral diagnosis should be segregated from all other specimens. The CSF specimen must be accompanied with simultaneously collected serum sample. One pair of specimens should be obtained as early as possible after the onset of concerned illness; the other pair of CSF and serum should be obtained 2-3 weeks later. These specimens obtained at two different occasions are then dealt together to demonstrate rise of antibody titre. In case of a tentative diagnosis of subacute sclerosing panencephalitis or multiple sclerosis a single pair of serum and CSF might be sufficient for testing against measles antibodies.

VIRUS ISOLATION

For viral isolation, the specimen must be obtained as early as possible after the onset of the clinical condition. The specimens must be obtained from multiple sites i.e. throat swab, urine, faeces, CSF etc. The specimens are to be transported in a Virus transport Medium (VTM). It is basically a buffer with balanced salt composition and bovine albumin stabilise the viruses. Antibiotics are added to keep the bacterial overgrowth in check. VTM is obtained from the virus laboratory according to the need or it can be prepared as described (see TRANSPORT MEDIA, Virus transport medium on page 73 for details). Such specimens must not be frozen and must be kept around 4°C. However, in case of delay these may be snap frozen at -70°C or transported in a container of liquid nitrogen or in dry ice. The viral isolation is done either on cell cultures or a laboratory animal; according to the clinical condition of a patient. The selection of the battery of most appropriate cell lines is essential. It should be noted that it takes many days to up to three weeks in the viral isolation. However, by detection of early antigens, the procedure might be expedited.

TESTS BASED ON DIRECT DETECTION

For direct detection of virus, the concentrated clinical material is transported quickly without any delay. No additive is used. Following are needed:

1. Nasopharyngeal aspirate for Respiratory Syncytial Virus
2. Vesicular fluid on a slide for Herpes simplex virus and Varicella Zoster virus
3. Faeces for Rota Virus
4. Brain in buffer for Rabies or Herpesvirus simplex

The transport must be quick and special logistic arrangements must be made in such cases. In cases of suspicion of dangerous pathogens, prior information must be provided to the laboratory. The nasopharyngeal aspirate must immediately be dealt without any delay to avoid the cell lysis. However, after the fixation of cells by acetone, the slide may be kept in refrigerator.

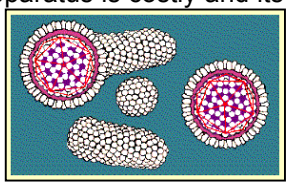
TESTS OF VIROLOGY USED IN BLOOD BANK

It is mandatory to test for Hepatitis B surface antigen (HBsAg) and anti-HCV. Only those samples, which are found to be HBsAg and anti HCV negative, are released for donation purpose. The test for Human Immune Deficiency Virus (HIV) is also done to prevent transmission of AIDS. Only those methods are to be followed which are easily adaptable at peripheral laboratories. The blood donations, which give doubtful or clearly positive results, are discarded. However, the donors are only told about their status when a reference laboratory duly confirms the test result. This information is handled with complete confidentiality and the laboratory record must not be made available to any unconcerned person.

Hepatitis B Surface Antigen (HBsAg)

Radioimmunoassay (RIA) and Enzyme linked Immunoassay (ELISA) are most sensitive and

best methods. The reagents for RIA have hazard of radioactivity, their half-life is limited, instrumentation is expensive and available only at few centres. ELISA apparatus is costly and its methodology is complicated. The alternatives are Reverse Passive Haemagglutination test (RPHA) for HBsAg.



Although these are less sensitive as compared with ELISA and RIA, they may still pick about 99% HBsAg positive donations. It is based on the principle that sensitised red blood cells (fixed chicken erythrocytes with adsorbed, highly purified guinea pig anti-HBs IgG immunoglobulin) are agglutinated specifically in the presence of HBsAg in the serum. The test procedure is simple, can be completed in about 1 hour and the result can be read without any instrument This test is mostly performed qualitatively but occasionally for the purpose of the titration of HBsAg in serum or a secretion, it can be adopted quantitatively. Commercial kits are available and their procedures have minor variations. Microplates of plastic (disposable) and 25 µl dropper are required. Buffer and reagents are provided in the kit.

Anti Hepatitis C Virus Antibody (Anti-HCV)

Enzyme linked Immunoassay (ELISA) is the best for diagnosis of Hepatitis C. There are various generations of ELISA tests. Serum or plasma sample is incubated in the wells coated with recombinant antigens of hepatitis C virus. HCV specific antibodies, if present, will bind to solid phase antigens, resulting in formation of antigen-antibody complexes. Enzyme labelled anti-human IgG is added which binds with the complexes, if present. After unbound enzyme labelled antibodies is removed by washing, a substrate solution is added. The presence of HCV specific antibodies is indicated by colour development. Where facility for ELISA is not available, a test based upon particle agglutination can be used. In this method, gelatin carrier particles are sensitised with recombinant antigens of hepatitis C virus. These sensitised particles are agglutinated by the presence of antibodies to HCV in serum/plasma.

Anti Human Immunodeficiency Virus Antibody (Anti-HIV)

The most suitable procedure for basic screening is ELISA and for confirmation another ELISA procedure based upon an independent principle is applied. In USA and some other countries, WESTERN BLOTTING is still used for confirmation. In blood banks where ELISA apparatus is not available, particle agglutination test for screening may be a reasonable alternative. In this method gelatin particles coated with HIV antigen are used. These particles are agglutinated by the presence of antibodies to HIV in serum or plasma specimens. In this procedure, fresh specimens are the best ones and stored specimens give discrepancies in the results. Those donations that are anti-HIV positive must be discarded but specimens of sera from these donors must be sent to the reference laboratory for their confirmation.

MEMBRANE IMMUNOASSAYS FOR HBsAg, ANTI-HCV AND ANTI-HIV

Where facility for ELISA is not available, test devices based upon membrane immunoassays are in use for screening of blood for HBsAg, anti HCV and anti-HIV. In qualitative membrane immunoassays, the membrane is coated with recombinant antigens or antibodies on the test line region of the device according to the nature of the test. During the test, the serum or plasma mixed with protein-A coated particles or conjugated dye, migrates on the membrane. A coloured line in the test region indicates a positive test result immunochromatographically. The test is validated by appearance of coloured line in the control region. The sensitivity and specificity of these immunoassays by different manufacturers is variable. The specimens found positive on initial screening by these devices should be confirmed by ELISA method.

POLYMERASE CHAIN REACTION (PCR)

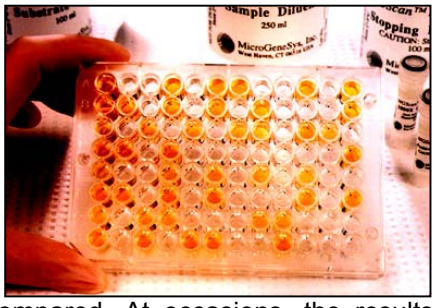
By PCR methodology, a fragment of the viral genome is multiplied to million-folds and subsequently detected. The procedure is currently done for Hepatitis C virus, Cytomegalovirus, Hepatitis B virus etc. In case of RNA viruses like Hepatitis C virus, the genome is extremely labile one and is quickly inactivated. Therefore, the specimen of serum is freshly obtained in the laboratory and quickly processed without any delay. While performing the procedure, contamination must be avoided and pipetting should be done carefully. For every specimen, at every procedure a separate, disposable pipette tip is used. The enzymes (i.e., Reverse Transcriptase and Taq polymerase) are extremely labile and must not be exposed to ambient temperature. These enzymes may be directly transferred while the vial is still in the freezer. The amplification of the

target nucleic acid is carried out using a thermocycler (Figure 29.2). This equipment provides successive cycles of varying temperatures, for various steps of PCR. The procedure is described in section on Molecular Techniques in Pathology on page 43 and MOLECULAR GENETICS, DNA analysis on page 300. During the process of the PCR, a continuous electric supply must be ensured by UPS (uninterrupted power supply system), connected to the thermocycler. Any power shut down will lead to disruption of amplification.

Figure 29.2: Thermocycler

ELISA TESTS

Enzyme linked immunosorbant assay (ELISA) procedure is useful for the diagnosis of viral diseases. It detects viral antigen (like HBsAg, Rotavirus and Respiratory Syncytial virus), IgG or total antibodies (i.e. Anti-HBc, Anti-HBe, Anti-HBs, Anti-HCV, Anti-HIV, Anti-Rubellavirus IgG etc.,) and some IgM antibodies (Rubellavirus, Hepatitis A virus anti-HBc IgM, Anti-HEV-IgM, Parvovirus IgM and anti-delta virus IgM). The ELISA apparatus is a modified colorimeter and is mostly designed in the form of a multi-welled plate reader. The intensity of developed colour in an individual well is measured and a computer printer prints the results. The colour developed on control wells (positive and negative ones) are used for the determination of cut-off point, on the basis of which the results of test wells are compared. At occasions, the results may be quantitatively measured. This is done for the determination of antiviral antibody titres in case of babies born with congenital infections for the CMV and Rubella. The decline in titre shows the original presence of passively transferred maternal antibodies and the stable or rising titre means differently. Moreover, these are required in case of those vaccinated against Rabiesvirus or Hepatitis B virus. This is done by serial dilutions of positive controls and plotting their results on a graph paper. In routine, ELISA tests are used for HBsAg, Anti-HCV and anti-HIV tests in blood banking and clinical laboratories. In case of indirect test, it is a three-step procedure, in case of a competitive ELISA it is two-step procedure. It takes up to four hours for the completion of the tests because of number of incubations. An extremely small quantity of the serum is required for ELISA tests (see also ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA) on page 225).



SYNDROMES IN VIROLOGY

Over a period of time, virology has become an importance field of laboratory medicine because of:

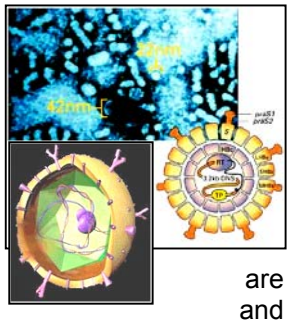
1. Discovery of more viruses and knowledge about their association with already existing clinical syndromes.
2. Appearance of new viral diseases likes AIDS, SARS etc.
3. Discovery of association of viruses with cancers.
4. Discovery and successful use of antiviral drugs.
5. Ever-expanding field of viral vaccines and their judicious use in eradication of certain viral diseases like smallpox in the past, poliomyelitis, and measles in current day situation.
6. Viruses and their role in congenital diseases.
7. Discovery of dreadful viral conditions like viral haemorrhagic fevers (i.e., CCHF, Lassa Fever virus, Marburg and Ebola virus).
8. Immunosuppressive therapy (as given in cancer and organ transplant recipients) with expanding horizon of opportunistic viral conditions.

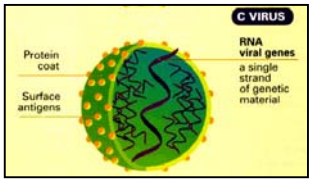
The number and pace of discoveries had been so rapid that most of the doctors and paramedical staff was unable to cope with them. Therefore, the selection of the most appropriate tests, the selection of types of samples and their time of collection are left mainly to the discretion of pathologist/virologists. However, a brief introduction to important viral syndromes is presented for general knowledge.

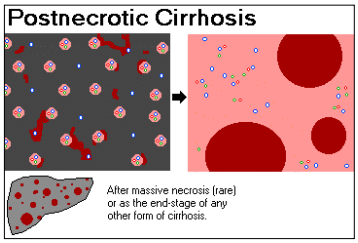
VIRAL HEPATITIS

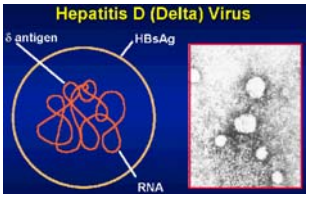
This may be acute or chronic (long term). It is the inflammation of liver, with worsening of

jaundice and decompensation of liver functions. These diseases are caused by viruses, which mainly affect the liver and include Hepatitis A to E viruses (HAV, HBV, HCV, HDV & HEV). HAV and HEV are transmitted by food and water and their disease is self-limiting. Once one is cured, there is no long-term effect on liver. Almost every one in our population acquires HAV infection before the age of 18 year, mainly without any clinical disease. Only 1/1000 persons gets signs and symptoms of disease but those who get the virus may pass it to other by their faeces and become permanently immune. The vaccine is available against HAV that is only recommended for children in the developed countries. Mainly the adults acquire HEV and the disease may be very serious in pregnant ladies in their last trimester. The HBV, HCV and HDV may be acquired asymptomatically but may persist in liver and cause chronic liver disease (CLD) with late complications like cirrhosis and even the liver cancer. The HBV is cleared by 95% of those who acquire it at adult age (in case the immune system is intact and functioning well). The HCV may persist in majority of those who are infected with it. These viruses are acquired by parental route; via blood and body secretions and the sharp reusable instruments contaminated with blood. The HBV causes symptomatic acute disease in only 30% of infected adults and seriousness of the disease varies from person to person. The HBV is sexually transmitted, as well as transmitted from mother to during childbirth. The HCV is not usually transmitted in such cases and only 3-4% of such persons are in danger. The HDV infects only those who are also infected with the HBV. Both these viruses if acquired may cause more serious disease. There is a vaccine available against the HBV, which also protects against the HDV. It takes many months for vaccine to be effective. Vaccination against hepatitis B is helpful in prevention. In case of needle stick injury with HBsAg positive blood or sexual exposure to HBsAg positive individual, immediate prophylaxis with vaccine and immunoglobulins is recommended, to non-vaccinated individuals. There is no need for testing all viral hepatitis markers in all cases. Their judicious selection is required which can be made on the basis of available clinical information. To avoid transmission of HAV and HEV, special emphasis should be on provision of clean food and drinking water. In case of HBV, HCV and HDV, the repeated use of needles, syringes lancets should be discouraged without autoclaving them. Medical and paramedical staff as well as their dental counterparts should adopt the safety precautions. The blood donors must be screened properly. The babies born to HBV carrier mothers should be protected at birth by administration of vaccine and specific immunoglobulins.









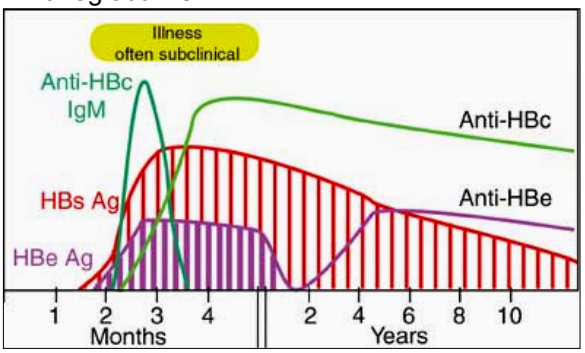
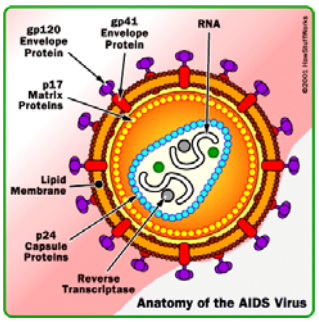


Figure 29.3: Clinical presentation of hepatitis

ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS)

This disease was not known before 1983 when for the first time, it was discovered in male homosexuals of USA. Human immunodeficiency virus (HIV) causes the disease. This virus affects the CD4+ T-lymphocytes (page 215) and nerve cells. The T-cells are decreased and after many years of HIV infection, the pool of these cells is exhausted and the body becomes defenceless against many types of



infections. These opportunistic infections from within the body and from outside may attack the person. Moreover, various kinds of cancers are also acquired. The HIV is transmitted by sex, blood transfusion, sharp instruments contaminated with infected blood and from mother to child. The virus remains in the body for many years and may be transmitted by these routes. However, by day to day contact and being in the same house or facility may not impose danger of HIV infection. The death occurs in case of all affected persons. Special care should be taken while dealing with blood and other laboratory specimens of all persons. Gloves and white coats must be worn and sharp instruments and needles must be handled with care. The surfaces, laboratory forms and other articles must not be soiled with blood. Ample quantity of hypochlorite solution must be used in the laboratory for decontamination. In case of rubber and metal items, 2% activated glutaraldehyde solution may be used for disinfection. The anti-HIV test is done by ELISA. In case of a positive test, it must be repeated on a fresh specimen and then it should be re-tested on a different kind of ELISA. However, Western blot confirmatory test is done in highly sophisticated laboratories and it is a gold standard. In case of babies born to HIV infected mothers, patients undergoing treatment and IgG deficient persons, PCR test for HIV RNA is recommended.

VIRAL HAEMORRHAGIC FEVERS

This syndrome is extremely dangerous because of nosocomial transmission to medical and laboratory staff and acute downhill course. In Pakistan Crimean Congo haemorrhagic fever (CCHF) occurs from time to time. The outbreaks are more common in Quetta and some other areas of Baluchistan. However, it may be found in any part of the country. The virus is transmitted by ticks and from blood and sharps used on patient. Minimum laboratory tests should be done and the patient should be isolated. Ribavirin may be used for prophylaxis and treatment during the early course of disease; no vaccine is available. The laboratory tests must be minimum and specimens must be dealt with care. Malaria, enteric fever and septicaemia should be excluded. The specimen should be transported in special double container with enough absorbent. They should be properly labelled and a prior contact should be made with the testing laboratory.

TORCH

This is misnomer and should better be avoided. It is used for **To** (Toxoplasma), **R** (Rubellavirus), **C** (Cytomegalovirus) and **H** (Herpes Simplex virus), It is considered that these three viruses and one parasite cause congenital disease. Herpes simplex virus does not cause the congenital syndrome. The congenital disease means that disease which is acquired from mother when the baby is still in the womb, especially during the early days of pregnancy. The tests are planned according to the clinical condition. These differ in case of pregnant ladies and babies of different age. These viruses do not cause repeated episodes of foetal loss/ damage and so called bad obstetric history. The Rubellavirus vaccine is available along with Mumps and Measles vaccine (MMR) and is routinely used in developed countries. In case of ladies, information must be made available about the duration of pregnancy (gestation), vaccinated or not vaccinated against Rubella and any previous baby affected. In case of baby, its age and congenital syndrome should be mentioned. After the age of six months it is not possible to offer appropriate diagnosis of congenital infections. In case of pregnant ladies (especially in their first trimester), special care should be taken in the collection of appropriate serum sample and performing the correct test as the termination of pregnancy may be advisable in affected cases with Rubellavirus.

SECTION V – IMMUNOLOGY

30. IMMUNOLOGY

Immunology is the study of immunity, a physiological process by which body protects itself from injurious agents, most of which are infectious organisms i.e., bacteria, viruses, protozoa, fungi etc. The immune system comprises complement system, cytokines, antibodies, phagocytes, lymphocytes, natural kill and antigen presenting cells. Immune system can recognise all potential threats by its inherent capability to differentiate between self and non-self. This differentiation depends upon receptors present on the surface of B-lymphocytes in the form of antibodies (sIg) and on the surface of T-lymphocytes as T-cell receptors (TCR). The immune system acts in three phases (Table 30.1). First phase is of **recognition.** It is accomplished with the help of B-lymphocyte receptors (surface immunoglobulins, sIg), and T-lymphocyte surface receptors. The second phase is of **activation** in which metabolic processes are activated inside the cells. The third phase is the **effector** phase. It follows phase of activation. In this phase the activated cells produce cytokines to activate other cells, differentiate into next phase (B-lymphocytes differentiate into plasma cells to produce antibodies), produce surface receptors and substances, which help in cytotoxic activity. Memory cells are also generated in this phase. The immune mechanisms are divided into two categories (Table 30.2):

- Nonspecific or innate immunity
- Specific or acquired immunity

Table 30.1: Phases and components of immune system.

	Antigen Recognition	Activation	Effects
B-lymphocytes	Surface Immunoglobulins (sIg)	Without T-cell help. Multiple combinations between antigenic sites and surface immunoglobulin (SIg) molecule	Differentiate into plasma cells. Generation of memory cells. Production of antibodies.
CD4+ Helper T-lymphocytes	With T-cell receptor only when antigen presented in combination with HLA class II molecule.	Initiated by TCR-HLA class II combination and requires activation of co-receptors and cytokines.	Cytokine production, T_H1 or T_H2; Generation of memory cells
CD8+ Cytotoxic T-lymphocytes	With T-cell receptors only when antigen presented in combination with HLA class I molecule.	Initiated by TCR-HLA class I activation. Requires activation of co-receptors and cytokines from Helper T-cells.	Cytotoxic activity; Apoptosis

Table 30.2: Features of Nonspecific and Specific Immunity

Feature	Nonspecific (Innate) Immunity	Specific (Acquired/Adoptive) Immunity
Characteristics		
Specificity for microbes	Low-Minimal	High
Diversity	Limited	Large
Specialisation	Low	Highly specialised
Memory	Nil	Present
Components		
Physical and Chemical Barriers	Skin, mucosal epithelia; anti-microbial chemicals in secretions such as defensins, lysozyme, acid in stomach, spermine etc.	Mucosal and cutaneous immune system and antibody molecules in secretions (secretory IgA)
Blood proteins	Complement and Cytokines (TNF, IFN gamma)	Antibodies (IgG, IgA, IgM, IgE, IgD), Cytokines
Cells	Phagocytes (Neutrophils, Macrophages, NK cells	Lymphocytes (B-lymphocytes, T-lymphocytes-Helper T-cells, Cytotoxic T-cells)

NONSPECIFIC (INNATE) IMMUNITY

The nonspecific immune mechanisms are also called innate as they act against all potential injurious agents in the same manner, even after repeated exposures. These mechanisms consist of the following:

Chemical and Mechanical barriers

The skin, mucosa (i.e., the lining of the gut, respiratory tract, urinary tract and the genital tract), hair in the nose and cilia in respiratory tract act as mechanical barriers while secretions on skin and mucosa like sebaceous secretions, lysozyme, mucus and acid in the stomach act as chemical barriers. Bacterial flora in different sites also act as inhibitors for the growth of potential pathogens (germs with potential to cause disease).

Humoral Factors

Humoral or fluid factors in the non-specific immune mechanisms mainly consist of complement proteins, interferon α, interferon β, tumour necrosis factor (TNF) and acute phase reactants like C reactive proteins.

Complement: Complement system consists of a series of proteins found in the plasma. These are activated in the form of a chain reaction

resulting in opsonisation, vasodilatation, chemotaxis, and formation of membrane attack complex (MAC). These proteins are produced by hepatocytes and macrophages and they are numbered from 1-9. In addition, proteins taking part in the activation of the alternate pathway are called factors. These factors are characterised by alphabets B and D (factor B and factor D). There are a number of control proteins, which are known by their function e.g., C1 estrase inhibitor (C1INH), decay accelerating factor (DAF) and homologous restriction fragment (HRF), or by the CD numbers assigned to them e.g., CD59 and CD55. Complement proteins act in a cascade or chain reaction. The complement activation can be initiated either by the classical pathway or by the alternate pathway (Figure 30.1). The antigen antibody complexes containing IgG or IgM in combination with the antigens initiate the **classical pathway** activation.

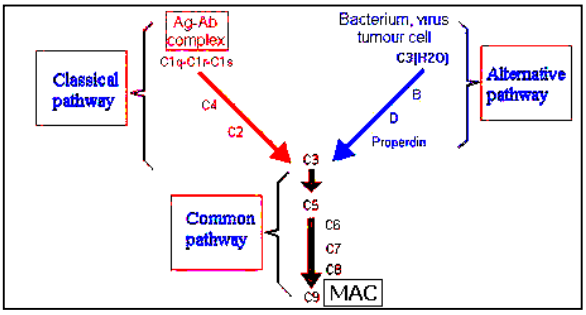


Figure 30.1: Pathways of complement activation

The classical pathway activation is classically observed in conditions where immune complexes are formed e.g., after infusion of foreign proteins like anti-snake venom serum raised in horses or in autoimmune diseases like systemic lupus erythematosis (SLE). The complement activation ultimately results in the formation of mediators called chemotaxins (C5a) and anaphylotoxins (C3a). Another important by-product of complement activation is the production of C3b. This helps in coating the target antigens (opsonisation) so that phagocytes, macrophages and neutrophils can easily eat up these target antigens (phagocytosis). The chemotaxins bring in the inflammatory cells like neutrophils to deal with organisms or other antigens and anaphylotoxins help in increasing the blood flow in the area of inflammation by causing vasodilatation. The ultimate result of the complement activation is the formation of membrane attack complexes (MAC) consisting of a combination of complement fragments C5b, C6, C7, C8 and C9. The MAC can physically produce holes in the membranes of the infectious organisms and other cell membranes. The complement activation can be measured in the laboratory by the assessment of C3 and C4 or by measuring CH50 classical pathway (in some places CH100 may be measured in place of CH50 depending on the technique being utilised). The classical findings in the immune complex mediated diseases would be decrease in C4, normal or slightly reduced C3 and reduced CH50. In some laboratories, the MAC can also be measured. This set of findings would be classical for SLE. It must be remembered that classical pathway requires formation of antibodies (IgG and IgM) for its activation. This would take some time (at least 7-10 days). So there is a need for a system, which can immediately bring all functions of the complement system into action (opsonisation, chemotaxis, anaphylaxis, formation of the MAC). This is achieved by the activation of the alternate pathway. The **alternate pathway** activation is always maintained at a low level, even in the healthy state, within the body. The presence of an antigenic surface, such as bacterial membrane, results in rapid activation of the alternate pathway.

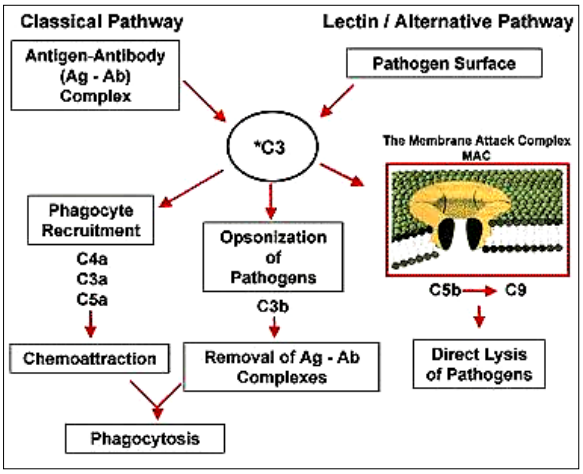


Figure 30.2: Results of complement activation

The classical findings of complement activation are normal C4 (which is low in classical pathway activation), decreased C3, normal CH50 classical pathway and low CH50 alternate pathway. The CH50 for the classical and alternate pathways can be separately measured with the use of sensitised rabbit RBCs for the classical pathway and non-sensitised guinea pig RBCs for the alternate pathway (a calcium chelating agent is also required such as EGTA with addition of magnesium in the form of magnesium chloride). The classical findings of the alternate pathway activation are observed in

post streptococcal glomerulonephritis.

Table 30.3: Interpretation of complement changes

Complement level				Example
CH50	C3	C4	Factor B	
Increases	Increased	Increased	Increased	Acute and chronic inflammation
Decreased	Decreased	Decreased	Normal or decreased	SLE, vasculitis
Decreased	Decreased	Normal	Decreased	Post - streptococcal glomerulo-nephritis
Decreased	Decreased	Normal	Normal	Hereditary angioedema

Cells

Phagocytes are cells, which can engulf particles of appropriate size in their cytoplasmic processes (phagocytosis). The target particles are later digested with the help of enzymes. This process is facilitated by opsonisation (coating by complement proteins or antibodies or, in a better way, by a combination of antibodies and complement proteins). Neutrophils and macrophages act as phagocytes.

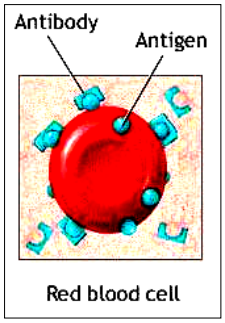
ACQUIRED IMMUNITY

Acquired/specific immunity is of two types. **Active**, which is generated when individual is exposed to antigen, and **Passive or adoptive transfer**. In this type the components of immune response, e.g., antibodies in serum are collected from a donor and transferred to a patient who acquires immediate immune response (such as anti-snake venom injected in a snake bite victim). The individual becomes immune for a short period of time without being exposed to the antigen. The following properties are specific for the acquired (specific) immune system (not found in nonspecific/innate immune system):

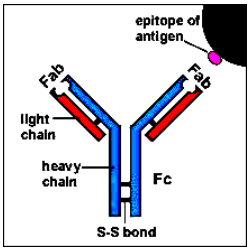
1. **Diversity**: The different types of receptors (variety of surface antibody molecules and T-cell receptors) available to differentiate between various injurious agents (mostly infectious organisms).
2. **Specificity**: Each type of the receptor has the capability to recognise and combine with only one target antigen. That is why this system has to maintain a variety of receptors.
3. **Memory**: The specific immune system maintains and increases the number of cells, which have come in contact with the target antigens. Thus, it remembers the potential threats. Frequent contacts would result in larger number of memory cells.
4. **Self-Regulation**: The system has an in-built mechanism of self-regulation to control the growth of immune effector cells (B and T-lymphocytes) after antigenic stimulation.

Mechanisms

The main mechanisms involved in specific immunity are through antibodies and T-lymphocytes. **Antibodies**: Antibodies are protein molecules found in blood. These are produced by plasma cells. Plasma cells are differentiated (developed) forms of B-lymphocytes. B-lymphocytes are produced in the bone marrow. These cells are also found in the germinal centres of lymphoid tissues like tonsils, spleen and lymph nodes. Antibodies are of five different types: IgG, IgA, IgM, IgD and IgE. Ig is the abbreviation for immunoglobulin while the letter G, A, M, D and E stand for the heavy chains in the antibody molecule (G for γ, A for α, M for μ, D for δ and E for ε). Antibodies are effective against antigens by their actions of opsonisation, activation of complement by combining with the antigens and forming immune complexes. Each type of antibody molecule can combine with only one type of the antigen (specificity). Some antigens become ineffective after the formation of immune complexes, neutralisation of toxins, and prevention of infection by bacteria and viruses. However, antibody molecules cannot reach inside the cells. That is why pathogens, which are able to survive inside the cells are protected from attack by the antibody molecules (e.g., Mycobacteria). Such pathogens and malignant cells are dealt with by the T-lymphocytes. Antibody molecules have two light and two heavy chains. The different antibody molecules (IgG, IgA, IgM, IgD & IgE) are identified by the differences in the heavy chain of the protein molecules. These different antibody molecules have some differences in function as well (see also Table 42.1). The above-mentioned varieties of immunoglobulins are also called antibody isotypes.

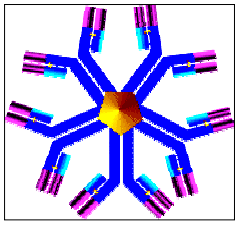


IgG: This is the antibody molecule, found in the highest concentration in the serum (5.1-16.1 g/L in adults). This antibody is produced relatively late after antigenic stimulation (secondary immune response). It persists for years after its appearance because of its half-life of about three weeks and its large concentration. It can



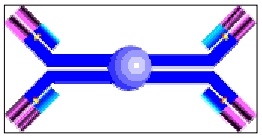
cross placental barrier so investigations based on the detection of this antibody would also be positive in a newborn if mother was positive. That is why the diagnosis of an infectious disease is more reliable in neonates if IgM, rather than IgG type of antibody, is detected. IgG with two complement binding sites is less efficient in fixing the complement than IgM antibody molecule, which has five complement binding sites. So IgG antibodies, in relatively less numbers, may be able to coat the target antigens but may not activate the complement. In this situation, it may block the combination of IgM molecules with the same antigen and thus "block" the action of IgM antibodies. The IgG type of antibodies is more efficient in precipitation reactions than agglutination based reactions. IgG antibodies are further subdivided into four subtypes based on the differences in heavy chains. These are called IgG subclasses: IgG1, IgG2, IgG3 and IgG4. Their relative concentrations are in the same proportion as their numbers; IgG1 found in the highest concentration. IgG2 subclass deficiency is found in about 40% of individuals who are IgA deficient. These individuals may suffer from recurrent chest infections and may also benefit from IgG replacement therapy. The IgG replacement has no role to play in IgA deficient individuals who do not have IgG subclass deficiency. However, replacement therapy in IgA+IgG2 subclass deficient individuals can be hazardous due to formation of anti-IgA antibodies in the patient leading to anaphylactic reaction as most of intravenous IgG preparations contain some IgA. IgG4 levels are increased in response to effective immunotherapy with allergens. IgG1 and the IgG3 subtypes are increased in response to protein antigens (diphtheria & tetanus) while IgG2 and IgG4 are increased in response to carbohydrate related antigens (meningococcus and pneumococcus). The IgG type of antibody is part of secondary immune response and it cannot be synthesised without help from CD4+, helper T-lymphocytes.

IgM: These antibodies are found as pentamers. That is like having five IgG antibody molecules joined at their bases with the help of a joining protein chain. This antibody molecule is the first one to be manufactured after the antigenic stimulation. It can be manufactured by B-lymphocytes without T-cell help particularly if

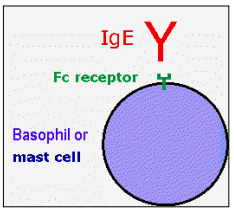


B-lymphocytes come across an antigen with repeat antigenic sites. This antibody has a short half-life of about 2-3 days. IgM disappears within 3-6 months after antigenic stimulation is switched off. That is why these antibodies are associated with active infection or infection in the recent past. IgM antibodies are efficient in complement fixation and agglutination reactions. These are found in relatively less concentration in serum (0.5-2.0 g/L in adults). These antibodies cannot cross placental barrier.

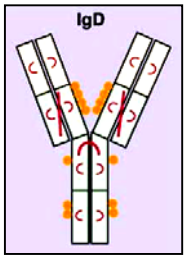
IgA: These antibodies are usually found as dimers like two molecules of IgG joined together. IgA is found on the mucosal surfaces because of a special protein attached to it called secretory piece. This antibody performs important protective function on the mucosal surfaces (GIT, respiratory tract, urinary tract, genital tract and conjunctival surface etc). This antibody cannot fix complement or cross the placental barrier. It is found in serum in concentration higher than IgM but less than IgG (0.8-4.0 g/L in adults).



IgE: These antibody molecules are special. They are produced in place of IgG as part of the secondary immune response against specific antigens. This would occur in individuals with special genes (atopic individuals).



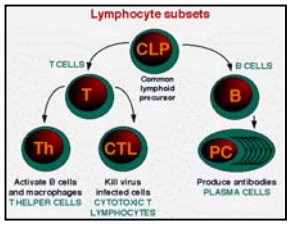
IgE antibodies have the ability to be caught by their Fc portions on the surface of the basophils and mast cells. On exposure to the antigens, these IgE molecules bunch together and activate the mast cells and basophils, resulting in release of histamine and other chemicals. These chemicals cause blood vessel dilatation and narrowing of airways producing typical manifestations of allergy. IgE antibodies have been shown to be active in immune response against parasites. However, their significance lies in allergy. They are found in relatively small concentrations in serum (less than 175 IU/ml in adults). In allergy, antigen specific or allergen specific IgE can be measured. These levels help in the identification of the allergen causing allergy in patients. The levels of allergen specific IgE decreases with effective immunotherapy.



IgD: These antibody molecules are found in almost negligible amounts in serum. Maturity of the B-lymphocytes is indicated

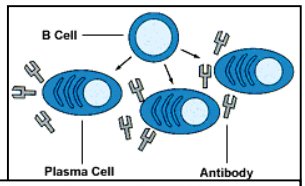
when IgD molecule appear on the surface of the B-lymphocytes along with IgM molecules. The immature B-lymphocytes only display IgM type of molecules on their surface.

Lymphocytes: The lymphocytes are mononuclear cells. The nucleus is rounded and only a thin rim of cytoplasm is visible. These cells cannot be differentiated on the basis of morphology. They are identified on the basis of protein receptors and the CD (Cluster of Differentiation) markers present on their surface. The lymphocytes are of following types:

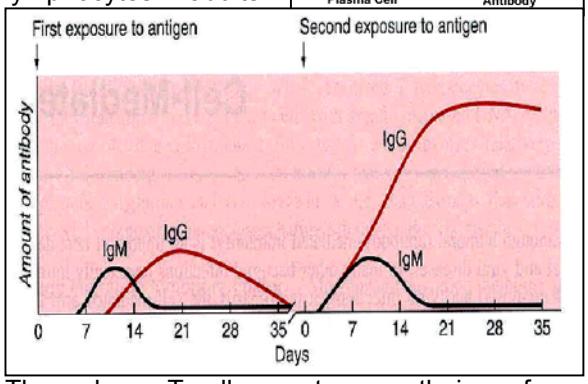


a. B lymphocytes
b. T lymphocytes
c. NK cells

B lymphocytes: These are concerned with the production of antibodies and form about 10-15% of the total lymphocytes in the peripheral blood in adults. In response to exposure to an antigen these are transformed to plasma cellsand produce various classes of antibodies in an orderly manner

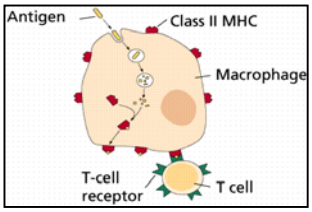


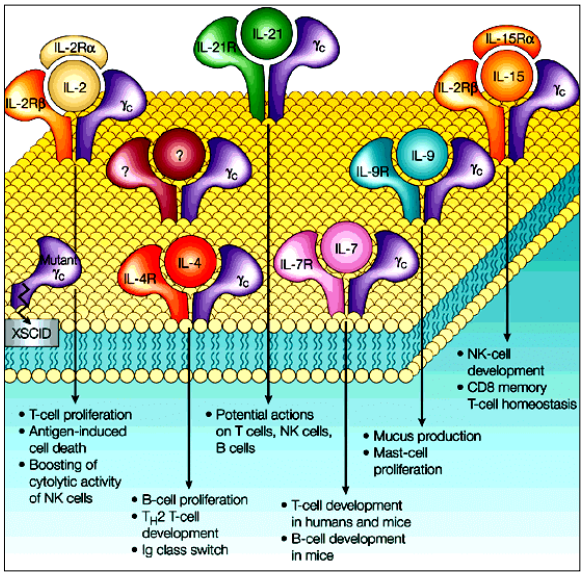
T lymphocytes: T-cells form the main component (70-80%) of the total lymphocytes in adults.



These have T-cell receptors on their surface. The T-cell receptors (TCR) are of two different types called TCR1 (with γ and δ protein chains) and the TCR2 (with α and β protein chains). The T-cells with TCR2 (α and β protein chains) form about 95% of the total T-lymphocytes in the peripheral blood. These are the T-cells (TCR2- α and β chains), which we will be discussing in relation to subtypes. The TCRs are used to feel and recognise the antigens. The antigens can only be recognised by the T-cells if they are presented to them after processing by the **antigen presenting cells** (APC). The antigen presenting cells, mostly macrophages, present the antigen in combination with the HLA molecules. The T-cell receptors are specific for the antigens. So each type of T-cell receptor can recognise only one type of antigen. T-lymphocytes can be recognised because of the TCR and CD3, CD2 and CD7 markers. These CD markers are also called pan T-markers. These are used in immunophenotyping reactions to identify T-lymphocytes. The predominant T-cell population is further divided into two subtypes. Helper/inducer T-lymphocytes (CD3+ CD4+ CD8- T-lymphocytes) are identified by the presence of the pan T-lymphocyte marker CD3 and the helper T-cell marker CD4 on their surface. These cells are also known in relation with HIV infection. HIV attacks the helper T-cell through the CD4 receptor. That is why in advanced HIV infection (AIDS), the CD4 positive helper T-cells are decreased in number (page 208).

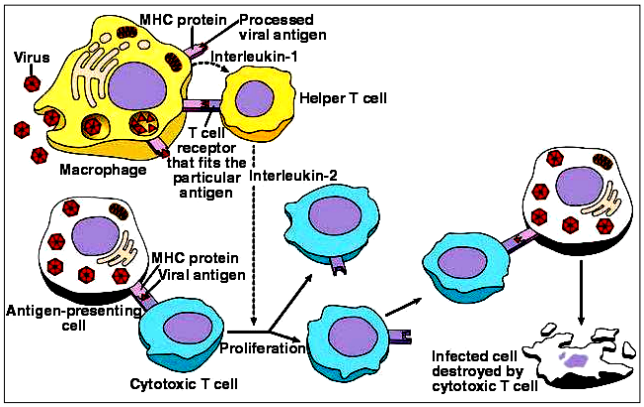
Helper T-cells form about two third of the total T-lymphocytes. The reference range for adults is considered as percentage of the total lymphocytes (38-46% in adults) and also in absolute numbers (0.7-1.1 X 10^9/L). Helper T-cells can recognise the antigen only when the antigen presenting cells in combination with HLA Class II molecules present it to them. Antigen combination with HLA class II molecules are possible when antigens are made available after phagocytosis. The antigens are phagocytosed usually after bacterial infections. Helper T-cells become





stimulated after recognising the antigen and start to produce proteins, which help in stimulation of other cells like B-lymphocytes, cytotoxic T-lymphocytes and the antigen presenting cells. The help from helper T-cells reaches other cells by way of **cytokines**. Cytokines are protein molecules and are also known as interleukins and lymphokines. Helper T-lymphocytes producing interferon γ and IL-2 promote cellular immunity. These cells are called T_H1 lymphocytes. Some helper T-cells may produce more of IL-4 and IL-10. These T-cells are called T_H2 lymphocytes and their cytokines promote antibody production by the B-lymphocytes.

Cytotoxic suppressor T-lymphocytes (CD3+ CD4- CD8+ T-lymphocytes) are recognised by the presence of pan T-lymphocyte marker on their surface in combination with CD8. Cytotoxic T-lymphocytes, as the name shows, act as the killer cells for the target cells. The target cells are the host cells, which have been infected and considered beyond repair by the immune system. The host cells may be affected in such a way either by viral infections or by malignant

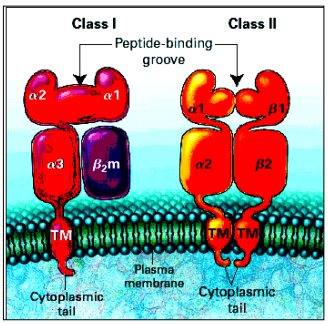


transformation. The cytotoxic T-lymphocytes can attack their target cells by coming in contact with receptors, which can induce a suicide within the target cell. These cells can also release chemicals that can punch holes in the membranes of target cell. Such a cell death is called apoptosis.

Natural Killer cells: These (NK cells, CD3- CD16+ CD56+) are the third type of lymphocytes. These cells are neither B-lymphocytes nor T-lymphocytes. Their exact site of development remains unknown. These cells are thought to play important role in anti-viral and anti-tumour immunity. They form 5-15% of the peripheral blood lymphocyte population and may be observed as large granular lymphocytes in the peripheral blood films. These cells tend to increase in chronic infections and autoimmune diseases.

HUMAN LEUCOCYTE ANTIGENS (HLA)

The human leucocyte antigens (HLA) are found on the surface of a variety of cells including leucocytes. HLA system is divided into two major classes: HLA Class I and HLA Class II antigens. The genes responsible for the formation of HLA are situated on the short arm of chromosome 6. The HLA Class I antigens are further divided into HLA-A, HLA-B and HLA-C subclasses. Each of these subclasses contains a number of antigens which are numbered as 1,2,3 etc (e.g., HLA A1, HLA B35, HLA C3). HLA class I antigens are found on the surface of all the nucleated cells and platelets as opposed to the HLA class II antigens which have a comparatively restricted distribution. The main function of the **HLA Class I antigens** is to present the antigens to the suppressor/cytotoxic subset of T-lymphocytes. T-lymphocytes cannot see/recognise the antigen unless it is presented to them in combination with the HLA antigens. CD8+ suppressor/cytotoxic T-lymphocytes can recognise the antigens when they are presented to them in combination with HLA class I antigens. HLA class I antigens assume a major role in initiating the cellular immune response in case of viral infections or when the cells are changed because of malignant transformation. **HLA Class II antigens** are subdivided into HLA-DR, HLA-DP and HLA-DQ groups. Individual antigens within these groups are numbered (HLA DR1, HLA DP2, HLA DQ3 etc.). These molecules present antigens to CD4+ helper/inducer T-lymphocytes. These antigens are prepared after phagocytosis by the macrophages. The helper T-lymphocytes are called “helper” because they start to produce the cytokines (Interferon γ, IL-2, IL-4, IL-10, IL-12 etc) after recognising the antigens in combination with the HLA class II molecules. These cytokines help macrophages and cytotoxic/suppressor T-lymphocytes to become more active in their functions. These cytokines are also the main driving force for the sensitised B-lymphocytes to produce antibodies required for the secondary immune response. The dependence of cellular and humoral (antibody) related function on the cytokines produced by helper T-lymphocytes makes these cells the



pivot in immune response. This importance is highlighted in HIV infection, which destroys the helper T-lymphocytes. In patients with advanced HIV infection, helper T-lymphocyte number is decreased and their function is impaired as well. This results in infection by opportunistic organisms and increased incidence of malignant disorders.

Importance of HLA in organ transplant

One of the main functions of the immune system is to differentiate self-tissues from all other kind of antigens. The immune system can recognise body's own tissues by the presence of HLA antigens on their surface. The T-lymphocytes have the inherent role of recognising the HLA antigens whenever they come in contact with anything. All cells displaying body's own HLA antigens are recognised as self and the T-lymphocytes pass on without getting activated. Thus immune response is not initiated. In organ transplant, tissue type (the combination of the HLA antigens; each individual usually carries six HLA class I and six HLA class II antigens) is determined by the tissue-typing. The tissue type of the recipient and the donor is matched so that when the donor organ is placed inside the recipient's body, the recipient's immune system finds the new organ as self and the immune response is not activated.

Importance of HLA in disease

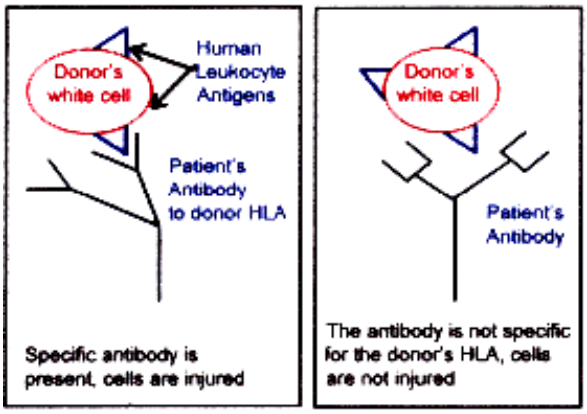
The immune system is largely activated after presentation of the antigens alongwith the HLA to the T-lymphocytes. That also indicates that if certain types of HLA antigens present more of one type of antigens some diseases would be produced either less or more in individuals having particular kind of the HLA antigens. The most significant HLA association is of HLA B27 with the development of the ankylosing spondylitis (80 times higher risk of developing the disease in HLA B27 positive individuals).

Importance of HLA in genetic identification of the individuals

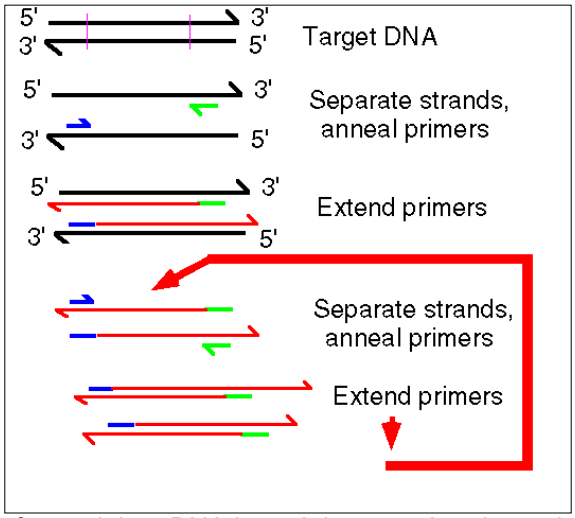
The large variety of HLA antigens in each subgroup and the biodiversity of the human population make a unique combination of the HLA antigens in one individual. This unique combination may be utilised for medico-legal purpose, though the importance in this respect has diminished with the discovery of other DNA markers.

METHODS OF DETECTION OF HLA ANTIGENS

HLA antigens can be detected by either serological methods or DNA based methods. In serological methods, lymphocytes are separated from the peripheral blood and made to react with a panel of antisera directed against all the



different HLA antigens. The combination of antibodies with HLA antigens on the surface of the lymphocytes is detected by cytotoxic reaction initiated by the addition of complement. The dead cells are then visualised under the microscope, with the help of dyes to assess the strength of the reaction. HLA class I antigens are detected on the surface of the T-lymphocytes while HLA class II antigens are detected on the surface of B-lymphocytes. These two lymphocytes are separated from each other with the help of Nylon Wool columns, monoclonal antibodies attached to magnetic beads or with the use of sheep erythrocytes forming rosettes with the T-lymphocytes in the classical reaction. The serological assays have been standardised as the microlymphocyto-toxicity assays. These reactions are carried out in the Terasaki trays, which can be read directly under the inverted phase contrast microscope



after staining. DNA based tissue typing depends on the use of DNA primers instead of antisera. These primers are sequence specific for the DNA genes responsible for the formation of

different types of the HLA molecules. DNA of the patient is extracted by phenol chloroform/ether extraction techniques and is adjusted for concentration. Then, it is incubated with the primers, in the presence of Taq polymerase, nucleotides and the required buffer, in a thermal cycler. The primers combine with the corresponding sequences and enhance the target DNA many times during the temperature cycles (Polymerase Chain Reaction or PCR, The enhanced DNA sequence is then visualised with the help of agarose gel electrophoresis or with the use of the fluorochromes. These methods require comparatively expensive equipment and reagents but the results of tissue type are more consistent and accurate in a carefully performed assay.

31. PRACTICAL PROCEDURES IN IMMUNOLOGY

HAEMAGGLUTINATION (HA) TESTS

Haemagglutination (HA) tests are used to detect antibodies or antigens. The test end point or the result is based on agglutination of the red blood cells (RBC) (Figure 31.1). The HA tests developed after the advances in the preservation of red cells, which may be stabilised by treatment with a number of chemicals to preserve their membrane integrity and surface adsorptive properties. Treatment of RBCs with tannic acid and/or glutaraldehyde improves the attachment of antigen or antibody and transforms them into sensitive agglutination test vehicles. Different types of haem-agglutination tests are:

1. Direct haemagglutination tests
2. Indirect haemagglutination tests
3. Reverse passive haem-agglutination tests
4. Haemagglutination inhibition tests

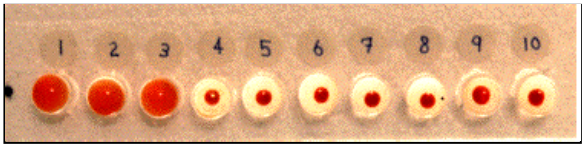


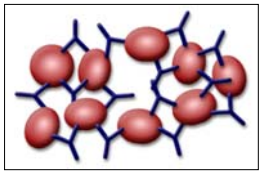
Figure 31.1: Appearance in haenagglutination test

DIRECT HAEMAGGLUTINATION

Red blood cells from various animal species may be clumped by certain viruses, which attach to their surface or the red blood cells may be agglutinated by corresponding blood group antibodies.

INDIRECT (PASSIVE) HAEMAGGLUTINATION TEST

In indirect haemagglutination test, known antigen is coated on to surface of red cells. These red cells are added to serial dilutions of patient serum and observed for either bead or carpet formation in the wells of the U bottom microtitration plates (Figure 31.2). Examples are treponema pallidum haemagglutination assay (TPHA), antibodies against amoeba, hydatid cyst etc. Positive and negative control sera should be included in each batch of test and treated as patient serum. Patient's serum should be heat inactivated at 56°C for 30 min in a water bath before performing a haemagglutination test. The test serum is serially diluted in buffer in the wells of a microtitre plate. Fixed amounts of sensitised red blood cells are added to each well. The plate is left at room temperature, out of direct sunlight and free from any vibrations. Reading is taken after 30-60 min. In a positive test, sensitised cells are agglutinated by antibody and settle to the bottom of the well as a diffuse carpet. In a negative test, cells settle as a small circle or as a compact button at the bottom of the well. The end point should be read as the highest dilution of the sample giving approximately 50% agglutination of the test cells.



HAEMAGGLUTINATION INHIBITION TEST

This is used to detect antibodies against arboviruses, influenza, measles and rubella viruses. These viruses are able to agglutinate red cells because they possess haemagglutinins on their outer surface. Patient's serum is mixed with the viral antigens. If the antibodies to the virus are present in the serum, they combine with viral antigens making themselves unavailable for binding to indicator RBCs. Positive test is thus indicated by non-agglutination of RBCs. If the antibodies are absent, the viral antigens will remain free to agglutinate the indicator RBCs. The negative test will, therefore, be indicated by agglutination of RBC (Figure 31.2).

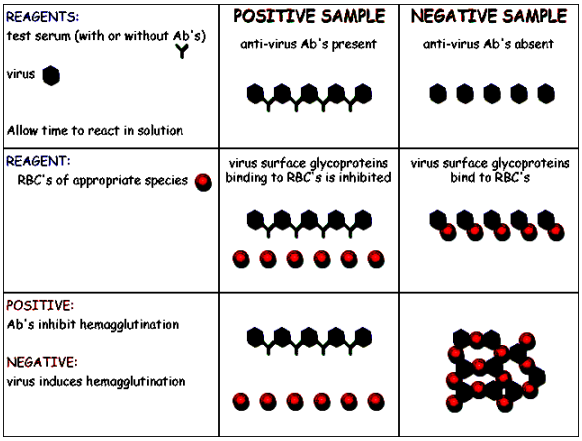


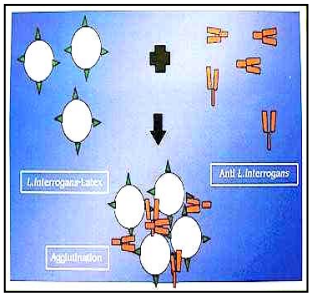
Figure 31.2: Details of haemagglutination inhibition test

REVERSE PASSIVE HAEMAGGLUTINATION TEST

It is used to identify antigens in the patient's serum. It is performed by incubating RBCs coated with viral antibodies with the patient's serum containing the viral antigen. If corresponding antigen is present in the serum, the RBCs will be agglutinated. Example is HBsAg detection.

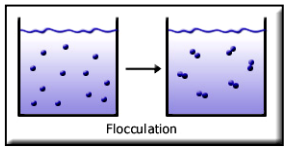
LATEX AGGLUTINATION TESTS

Latex particles can be coated with antigens or antibodies. These particles form a suspension on their own but form agglutinates, which are visible to the naked eye, when combined with antibodies or antigens. The latex particles are better visualised on dark surfaces, therefore, dark coloured glass slides or plastic cards are used to observe the antigen-antibody reactions. The latex agglutination reactions are easy to carry out. However, it must be ensured that the latex suspension does not show auto-agglutination due to improper storage or manufacturing fault. This can be easily checked by placing a drop of the latex reagent on the glass slide or the plastic card provided for the test. This drop is then spread out in the prescribed area and the card slide is gently rotated to look for auto-agglutination. The same method of rotation is used when the test/control sera are mixed with the latex suspension. The agglutination reaction is read after the prescribed time only, which is usually two minutes. A stopwatch must be used to read results at the correct time. Early reading may result in false negative results whereas late reading may give false positive results. This technique is used in several common tests e.g., pregnancy test.



FLOCCULATION TESTS

Flocculation is a phenomenon exhibited when antibodies (in a serum) are mixed with an antigen (in a suspension) in optimal proportions. It results in formation of floccules, which are visible to naked eye or with a hand lens. This phenomenon was most efficiently utilised in devising Venereal Diseases Research Laboratory (VDRL) Test. This test utilises a mixture of cardiolipin and lecithin as antigen suspension, which react with non-specific antibodies produced during the course of syphilis. The test has now been replaced with rapid plasma reagin (RPR) slide flocculation test. The antigen is coated on carbon particles. The sensitivity and specificity of this test is equivalent to that of VDRL but the results are easier to read.



COMPLEMENT FIXATION TEST (CFT)

Complement fixation tests make use of two properties of complement system:

- It is bound or fixed in antigen antibody (Ag/Ab) reactions, thus free complement is removed from the test system.
- Complement is required to haemolyse the sensitised RBCs.

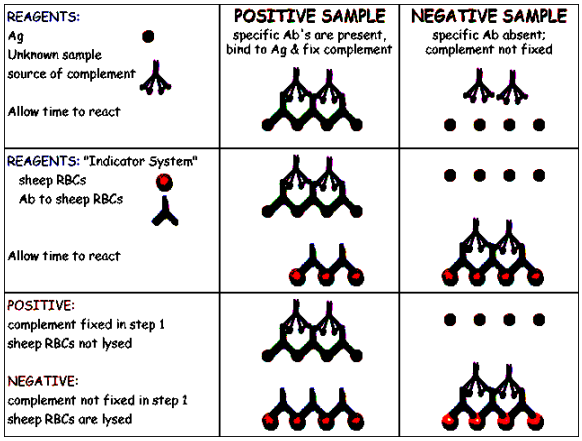


Figure 31.3: Details of complement fixation test

CFT consists of two reactions: non-haemolytic Ag-Ab reaction and the haemolytic indicator reaction. The first reaction involves antigen and its corresponding antibody and the latter reaction consists of RBCs and a lytic homologue, anti-erythrocytic antibody (haemolysin). Complement takes part in both reactions. Initially, patient's serum is incubated with the antigen. Then complement is added (Figure 31.3). If patient's serum contains antibodies, Ag-Ab reaction takes place, which fixes (removes) the complement from the test system. In the next reaction, sensitised RBCs are added. If complement is not available (used up in the first part of the test when antigens and antibodies combine to form immune complexes incorporating complement) it will not affect sensitised RBCs. If the complement is not removed in the first reaction, free complement remains available to react in second phase where it lyses the sensitised RBCs. The presence of haemolysis indicates that

complement is not consumed in the test system and the test is, therefore, negative. Absence of haemolysis indicates that the complement has been consumed in the Ag-Ab reaction of the test system and is, thus, not available for the haemolytic system. The test is, therefore, reported as positive. CFT is a preferred method for the serologic diagnosis of infections by *Mycoplasma pneumoniae*, Blastomyces, Histoplasma and most viruses.

Procedure

1. The wells of a microtitre plate are coated with an antigen.
2. Patient's serum is de-complemented by heating in a water bath at 56°C for 30 min.
3. Dilutions of the patient's serum and control are prepared and transferred to wells of microtitre plate.
4. Fixed amount of standard guinea pig complement is added.
5. The plate is incubated at 37°C or in a refrigerator, depending upon the specifications, to allow antigen antibody reaction and fixation of complement to take place.
6. Sensitised sheep RBCs are added and the mixture is incubated in 37°C for 30 min.
7. The plate is examined for evidence of haemolysis by a special visualising mirror.

Result

Positive tests show no haemolysis and the RBCs form a button at the bottom of the well. The negative tests show haemolysis and no button is formed. The test well showing 50% haemolysis is the titre of the antibody in the serum.

IMMUNOELECTROPHORESIS

Principle

Immunoelectrophoresis is a test procedure, which combines electrophoresis, diffusion and precipitation. It is used for immunochemical identification of abnormal protein bands detected by electrophoresis. The sample is first electrophoresed and fractions are allowed to interact with the corresponding antibodies deposited in troughs. Diffusion of antigens and antibodies towards each other forms antigen antibody complexes that are seen as precipitation lines (arcs) each representing one specific protein.

Buffer for immunoelectrophoresis (pH 8.6)

1. Sodium barbitone	15.45 g
2. Boric acid	14.25 g
3. 5,5,Diethyl barbituric acid	02.82 g
4. Sodium hydroxide	02.67 g
5. Distilled water up to	3 l
6. Sodium azide as preservative	0.3 g

Ponceau S stain: 300 mg Ponceau S stain is dissolved in 100 ml 5% acetic acid

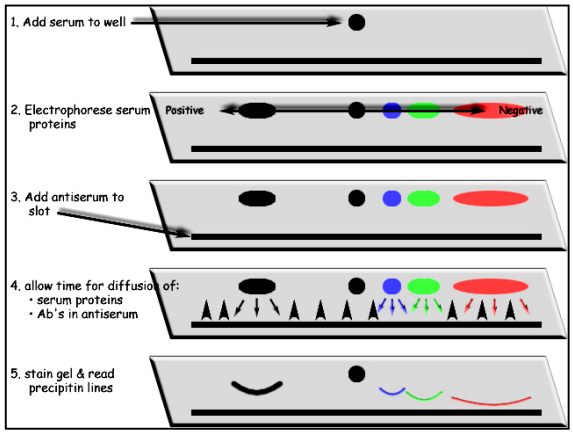


Figure 31.4: Procedure of immunoelectrophoresis

Procedure

This is for detection of paraproteins in serum or concentrated urine specimens.

1. Prepare 1% agarose gel in barbitone buffer and spread evenly on the support medium (Gelbond). Kits provide prepared gels.
2. Stain an aliquot of serum sample with bromophenol blue.
3. Make antigen slits in the gel by an applicator.
4. Remove excess water from the slits with a filter paper and dispense 2 µl sample (patient's serum alternating with controls) in each slit.
5. Electrophoresis is carried out at 180 volts for 45 min. Examine periodically the stain front. It should not move onto the filter paper wick on the edge of the gel. A total migration, from application to dye front, of 3.0-3.5 cm is enough.
6. After electrophoresis, cut two uppermost lanes and put in the stain fixative (Ponceau S stain in acetic acid), to be used as a reference strip.
7. Make troughs between the alternating control and patient samples with the help of a cutter.
8. Dispense 20 µl antibodies (IgG, IgA, IgM, κ and λ) in corresponding troughs.
9. Incubate for 18 hours at 4°C in moist chamber.
10. Wash in 2-3 changes of normal saline for 24 hours.
11. Dry the strips and stain with Ponceau S stain, destain to study precipitin lines.
12. Compare the control and patient's serum for each antibody. Abnormal arcs are identified

by their shape and location, present in a position corresponding to the homogeneous protein band in the reference strip (Figure 31.5).

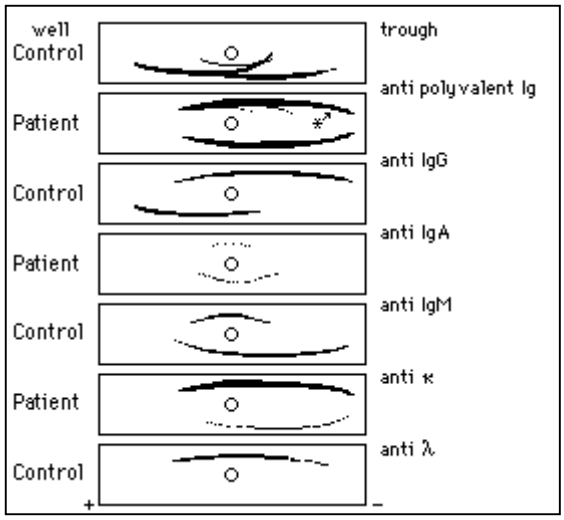


Figure 31.5: Results of immunoelectrophoresis

COUNTERCURRENT IMMUNOELECTROPHORESIS (CIE)

CIE is utilised to detect the antigen-antibody complexes in a precipitation reaction after electrophoresis in agarose gel. Only IgG type of antibodies can be detected. CIE depends on the property of IgG molecules having minimal negative or neutral charge on them. Most of these IgG molecules are made neutral by using a modified barbitone buffer. In the conventional set up, test sera are dispensed in wells cut into agarose gel, while the antigen is placed in a trough cut opposite to the wells (Figure 31.6). After electrophoresis, the gel is left at 4°C in a moist box overnight to facilitate precipitation. The antigen-antibody complexes are visible as arcs. These may be better studied if the gel is washed, dried and stained. This test system may be utilised to detect anti-Extractable Nuclear Antigen antibodies (anti-ENA antibodies).

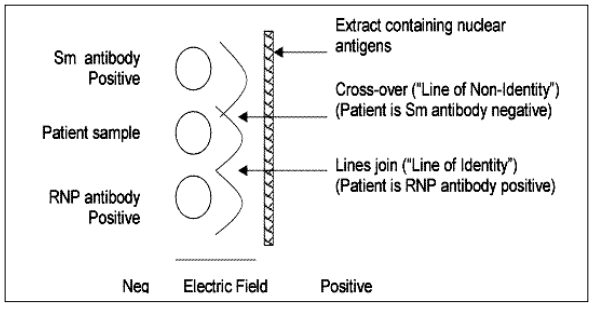


Figure 31.6: Countercurrent immunoelectrophoresis (CIE)

IMMUNOFIXATION

Immunofixation is the recommended method for the immunochemical identification of abnormal proteins detected by electrophoresis. It is quicker, simpler to interpret and more sensitive than immunoelectrophoresis. However, it is likely to be false negative in case of paraproteins being present in very high or very low concentration.

Principle

Electrophoresis of the sample is performed in six lanes. With the help of an applicator, about 75 µl of each antisera (anti IgG, IgA, IgM, κ and λ) are applied. The sixth lane is used as a reference strip. After cutting and fixation in protein fixative, these are examined for the presence of bands. The appearance of protein bands in the respective lane (designated according to the antibody reagent applied) helps in identification of the paraproteins in the test specimen.

Procedure

- Prepare 1% agarose gel in barbitone buffer and pour on a glass plate. Commercially prepared gels are also available.
- Patient's serum is applied in six lanes with the help of an applicator.
- Electrophorese for 30 min at 150 volts (voltage and time varies according to the gel). Paint each lane with one antiserum (anti-IgG, IgA, IgM, κ and λ). The sixth lane is painted with protein fixative such as acetic acid. Commercial kits provide a plastic template for this purpose.
- Electrophorese again for five min at 10 volts.
- Wash the strip in two changes of saline for 15 min each.
- Dry the strip, and Stain with Ponceau S.

RADIAL IMMUNODIFFUSION (RID)

Radial immunodiffusion is performed to detect the precipitating antigen-antibody complexes in a qualitative or quantitative manner. It may be utilised to quantitate immunoglobulins (IgG, IgA, IgM) and components of complement (C3, C4) in the serum. In its simplest form, it is called **Mancini technique**, which may be performed with Fahey's modification. RID may be used by Ouchterlony method for the identification of antibodies.

Principle

Mancini technique: The specific antibody is incorporated in agarose. The antigen (in the serum) diffuses radially and produces a ring of precipitation. The area enclosed by the ring is

proportional to the concentration of the antigen (in the serum) provided the diffusion proceeds to completion. The square of the diameter of ring (D^2) is proportional to the area of a circle so a plot of D^2 against antigen concentration will be a straight line (Figure 31.7).

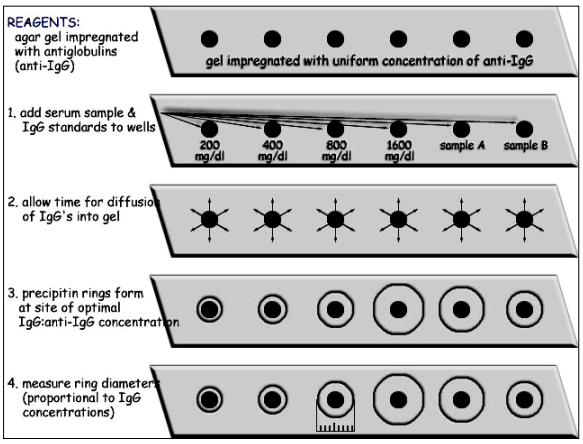


Figure 31.7: Radial immunodiffusion (RID) technique

Procedure

- Prepare 0.8% agarose gel and add polyethylene glycol 600 (1%) to enhance precipitation.
- Place the flask (containing agarose), cups, tips, plates, and pipettes in water bath at 56°C for 15 min and add antibodies to agarose; IgG: 200 µl to 10 ml gel, IgA, IgM, C3, C4: 100 µl to 10 ml gel.
- Mix and pour the gel into the plate. Store at 4°C in a moist box.
- Note the numbering system of the wells on the bottom of the plates and prepare worksheet accordingly to identify each test serum, control and standards.
- Punch holes in the gel, 1 cm apart.
- Shake the test sera and deposit 5 µl in respective wells in the plate, using separate tips for each serum. Control and standards are added similarly.
- Plates are placed upside down in a moist box and kept in the dark at room temperature.
- Measure the diameters of the precipitation rings for small molecules after 72 hours and for large molecules after 96 hours.

Results

Squares of the ring diameter (D^2) are plotted against the known concentrations of the standards. A straight line is obtained by joining at least three points. The concentration of test sera is read from this curve. In Fahey's modification, D^2 is plotted against the log of the concentration taking reading at 18 hours for small molecules.

ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA)

ELISA is based on the principle that one immune reagent can be immobilised on the solid surface while retaining its activity and the reciprocal immune reagent can be linked to an enzyme in such a manner that both enzymatic reactivity and the immuno reactivity of this conjugate are retained. Solid phase assay requires immobilisation of antigens or antibodies on solid surface. Most ELISA formats require covalent coupling of enzyme to an antibody or antigen. The enzyme commonly used is horseradish peroxidase.

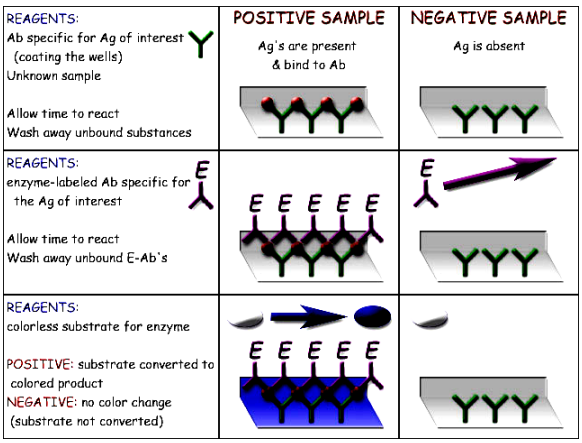


Figure 31.8: Details of antigen detection by ELISA technique

ANTIGEN DETECTION BY ELISA

ELISA is used for detection of bacterial, viral or parasite related or other types of antigens (Figure 31.8). These are of two types:

1. **Direct ELISA**: In direct ELISA, antigen specific antibody is attached to a solid phase. The test specimen is added followed by enzyme labelled antibody and chromogenic enzyme substrate.
2. **Indirect (antibody capture) ELISA**: Specific antibody is attached to a solid phase and test specimen is added to it. Specific antibody prepared in a species different from that coated on solid phase is added to combine with the antigen. Enzyme labelled antiglobulin specific for the second antibody is added. Chromogenic enzyme substrate is added and results are determined as for the direct ELISA.

ANTIBODY DETECTION BY ELISA

There are two methods:

- **Non-competitive ELISA**: Specific antigen is attached to solid phase by passive adsorption or with antigen specific antibody. Test serum containing specific antibody is added. Enzyme labelled antiglobulin specific for the test serum is added. Chromogenic enzyme substrate is added. The colour developed is proportional to the amount of antibody present in the test serum (Figure 31.9).

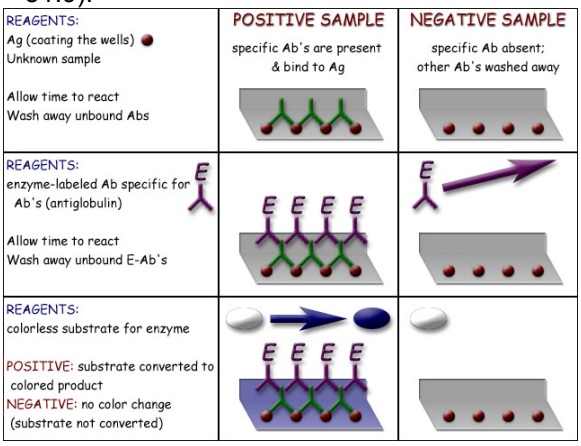


Figure 31.9: Non-competitive ELISA

1. **Competitive ELISA**: Antigen is attached on solid phase as for the non-competitive assay. The test serum and an enzyme labelled antibody specific for the attached antigen are added together. Chromogenic enzyme substrate is added. The colour developed is inversely proportional to the amount of antibody present (Figure 31.10).

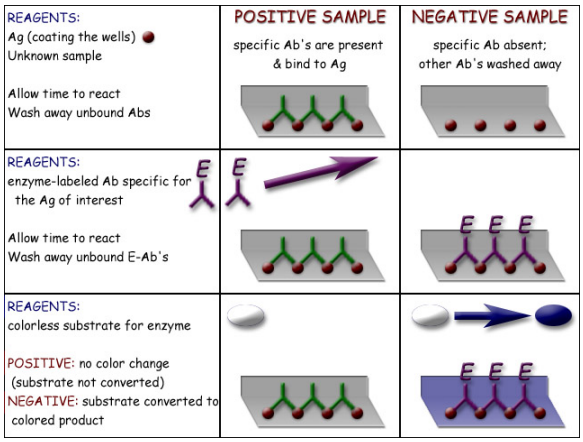


Figure 31.10: Competitive ELISA

FLOWCYTOMETRY

A fluorescence-activated cell scanner (FACS), also known as a flowcytometer, quantitates the fluorescence on individual labelled cells at a rapid rate, hundreds to thousands of cells per second (an impossible measurement using a fluorescence microscope). Although expensive to purchase, results are obtained quickly and easily in experienced hands, once the machine is set up. The cell suspension is incubated with appropriate monoclonal antibodies conjugated with a flourochrome. The cells are then washed to remove excess of antibody. The cell suspension is made to flow in a single cell file past a laser light source and light detectors. Fluorescent dyes are excited on the cell surface and fluorescence sensors detect emitted light (Figure 31.11). Light scatter can be measured to reflect the cell size and granularity. The data is expressed as profile histogram or dot plots. Usually whole blood collected in EDTA is required for the procedure. In case of leukaemias, bone marrow and in case of lymphomas, fine needle aspirate may also be used. The technique may be utilised to detect fastidious organisms in clinical specimens if appropriate conjugated antibody is available. It has also been utilised to study cell viability, nuclear ploidy and detection of preformed antibodies in cross match procedures before renal transplant. It can also be used to determine lymphocyte subsets, immunophenotyping of leukaemia and lymphoma, CD34, CD59 and HLA B27 assays. Flowcytometry remains a specific, sensitive and expensive technique, which is considered essential in good centres performing immunophenotyping.

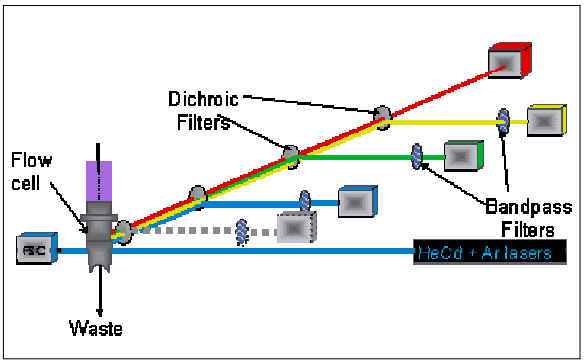


Figure 31.11: Schematic diagram of flowcytometry

Requirements

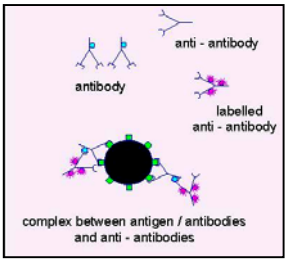
- EDTA container
- Fluorescence conjugated monoclonal antibodies
- Falcon tubes
- FACSLyse solution
- Centrifuge
- RPMI 1640
- 1% Formalin

Procedure

1. Draw 3 ml whole blood/0.5 ml bone marrow.
2. Obtain TLC, DLC.
3. Carefully check the antibody panel required for the procedure.
4. Label each test tube (Falcon, BD) properly and place in sequence. Put 10 µl of antibody in each tube as per defined panel.
5. Add 100 µl of whole blood/diluted bone marrow in each tube and mix it thoroughly.
6. Incubate in dark for 10 minutes at room temperature.
7. Make 1:10 dilution of FACSLyse solution in distilled water.
8. Add 2.0 ml of diluted FACSLyse in each tube.
9. Incubate in dark for 10 minutes at room temperature.
10. Centrifuge at 300g for 5 minutes at room temperature.
11. Discard the supernatant and shake the remaining 50 µl fluid to re-suspend the cells.
12. Add 2 ml RPMI 1640/PBS to each tune.
13. Centrifuge at 300g for 5 minutes.
14. Discard the supernatant and shake the remaining fluid.
15. Add 0.5 ml of 1% formalin to each test tube.
16. Keep at 4°C till analysis on flowcytometer.

IMMUNOFLUORESCENCE

Fluorescence is the emission of light of one colour (wavelength) while a substance is targeted with light of a high-energy wavelength (usually UV). High sensitivity and specificity makes immuno-fluorescence very useful in laboratory practice. Frozen sections from a composite block of several tissues, rat kidney, liver, and stomach are the usual substrates used to detect nuclear, gastric parietal cell, mitochondrial, smooth muscle and reticulin autoantibodies. There are two types of immunofluorescence techniques:



INDIRECT IMMUNOFLUORESCENCE

The antigen substrate (known antigen), usually in the form of frozen section or suspension, is applied to the slide. It is treated with patient serum. Antibodies in the patient's serum bind to the antigen. After washing in buffer, FITC conjugated anti-human antibody is added to the slide. If antibodies are present in patient's serum, they will bind to the target antigen (known antigen) and thus remain immobilised on the slide. In the next step conjugated antihuman antibody is added, which then binds to the antibody already bound to the antigen coated on the slide. The conjugated antibody will bind it and fluorescence can be detected by fluorescence microscope. The procedure is performed to detect autoantibodies in serum of the patient e.g., ANA, anti-ds DNA antibodies etc (Table 31.1).

Requirements

- Phosphate buffered saline (PBS) tablets
- Slides with tissue sections
- FITC Conjugates (IgG/IgM)
- Moist box
- Wash box
- Micropipettes
- Micro tips (disposable)
- Squeezable bottles/Pasteur pipettes (plastic)
- Test tubes
- Eppendorf tubes
- Black marker

Procedure

1. Inactivate all control and test sera at 56°C for 30 minutes.
2. Prepare 1:10 dilutions of the sera in PBS. Leave the pipette tips in the dilution tubes for later use.
3. Take out slides from the freezer; do not open the foil cover and keep at room temperature for 15 minutes to avoid water condensation.
4. Encircle the tissues section on the slide with a black marker.
5. Dispense 15 µl of test/control sera on the respective sections according to the worksheet; take extreme care not to mix the sera.
6. Incubate at room temperature for 20 minutes in a moist chamber.
7. Rinse with PBS.
8. Keep in PBS for 20 minutes at room temperature in a dark place.
9. Take the slides out of the box; blot the excess fluid; do not dry the sections; renew the slide markings.
10. Dilute the conjugated antihuman antibody 1/30 (or as recommended); dispense in 15 µl on each section.
11. Place cover slips and arrange the slides in a folder; keep the slide folder in refrigerator at 4°C.
12. Incubate in moist chamber at room temperature in a dark place.

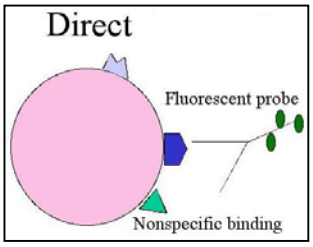
13. Rinse in PBS.
14. Keep in PBS for 20 minutes at room temperature in a dark place.
15. Take the slides out of the box; blot the excess fluid; do not dry the sections.
16. Place 1-2 drops of mounting medium (1:10 glycerol in PBS) on the slide.
17. Observe under the fluorescence microscope in the dark room.

Table 31.1: Parameters and corresponding substrate

Parameter	Substrate	Conjugates[1]
ANA	HEp2, liver/kidney (Rat)	IgG FITC
ASMA	Kidney (Rat)	do
Anti-centromere antibody	HEp2, Vero cell	do
Anti-mitochondrial antibody	Liver/kidney Rat)	do
Anti-skeletal muscle antibody	Thigh muscle (Rat)	do
Anti-liver/kidney microsomal antibody	Liver/kidney (Rat)	do
Anti-ds DNA antibody	*Crithidia luciliae*	do
ANCA	Human neutrophils	do
Anti-parietal cell antibody	Stomach (Rat)	do
Anti-histone antibody	Liver (Rat) 10N HCl)	do
Ant-adrenal antibody	Adrenal (human)	do
Anti-reticulin/gliadin antibody	Liver (Rat)	IgA FITC
FTA	*Treponema pallidum*	IgM FITC

DIRECT IMMUNOFLUORESCENCE

Direct immunofluorescence is carried out to detect the antigens (which may be trapped in immune complexes in tissue) with the help of conjugated antibody. The unknown antigen is fixed on the slide as frozen section, as in case of renal and skin biopsies or smears of clinical specimens may be examined for possible bacterial, viral or fungal pathogens. The slides are covered with the conjugated antibodies, incubated in dark for 20 min, followed by wash in normal saline for 20 min, (in dark). The slides are then mounted in aqueous mounting medium and studied under fluorescence microscope. The technique may be utilised for rapid diagnosis of microbial infections, especially when fastidious pathogens are suspected e.g., Legionella infection. It is also use for dealing with skin and renal biopsies received fresh, unfixed and in saline.



Requirements

- Phosphate buffered saline (PBS) tablets.
- Multi-spot slides with tissue sections.
- FITC Conjugates (IgG, IgA, IgM, C_3, C_4, Fibrin).
- Moist box
- Wash box
- Micropipettes and disposable tips
- Squeezable bottles/Pasteur pipettes (plastic)
- Test tubes
- Eppendorf tubes
- Black marker

Procedure

1. The tissue must be processed immediately or snap frozen in liquid nitrogen in OCT compound and stored at or below -40°C.
2. Cut multiple 5 µm sections on a tissue cryostat (six sections per slide).
3. Air dry for at least 30 minutes at room temperature.
4. Stain two slides for each biopsy specimen.
5. Encircle the tissue sections on the slides with a black marker.
6. Dilute 1:70 (or as required as determined by chequerboard titration) each FITC conjugated antihuman antibody with PBS.
7. Overlay with 15 µl of appropriate FITC conjugated antisera (against IgG, IgM, IgA, C3, C4 and fibrin) on the respective section.
8. Incubate in moist chamber at room temperature for 20 minutes.
9. Rinse in PBS.
10. Keep in PBS for 20 minutes at room temperature in a dark place. Longer washes will reduce the intensity of background fluorescence and are recommended.
11. Take the slides out of the box; blot the excess fluid; do not dry the sections; renew the slide markings.
12. Place 1-2 drops of mounting medium (1:10 diluted glycerol in PBS) on the slide.
13. Place the cover slips and arrange the slides in a folder; keep the slide folder in refrigerator at 4°C.
14. Examine slides under a fluorescent microscope.

HLA TYPING (COMPLEMENT MEDIATED MICROLYMPHOCYTOTOXICITY

It is used for identification of HLA antigens of recipient and potential donors for solid organ and bone marrow transplants, forensic medicine and disease association.

Requirements

Reagents

- HLA class I and II antisera (BAG,

[1] IgM conjugate may be used when IgM specific antibodies need to be detected.

Germany)
- Histopaque 1077 (ICN)
- RPMI 1640 (ICN)
- Sucrose powder (Merck)
- Eosin (Sigma)
- Formalin (Sigma)
- PBS tables (ICN)
- Rabbit complement (BAG, Germany)
- Nylon wool (Robbins)

Equipment
- Inverted phase microscope
- Hamilton syringe with dispenser
- Heparinised tubes
- Venoject needles
- HLA trays
- Centrifuge
- Test tubes
- Pipettes

Procedure

The test is carried out by using Tarasaki plates prepared with commercial HLA antisera. Ready-made HLA class I trays by one Lambda (USA) and BAG (Germany) are also used in special cases.

1. Draw 20 ml fresh blood in two 10 ml heparinised tubes. Mix well. Keep at room temperature.
2. Dilute fresh heparinised blood with equal volumes of RPMI 1640 with 5% foetal calf serum.
3. Dispense 4 ml histopaque in four tubes for each sample.
4. Dispense equal volumes of diluted blood over the histopaque in each tube. Take care not to mix the blood and histopaque.
5. Centrifuge the tubes at 1800 rpm (525g) for 30 minutes at room temperature. The lymphocytes will settle as a white ring at the boundary between the plasma and histopaque.
6. Using a Pasteur pipette, carefully aspirate the ring of lymphocytes and transfer to fresh plain tubes.
7. Fill the tubes with RPMI 1640 with 5% foetal calf serum. Mix well.
8. Centrifuge the tubes for 15 minutes at 1800 rpm (525g) at room temperature.
9. Discard upper layer. Re-suspend the deposit with RPMI 1640 to make 5 ml in each tube.
10. Dispense 5 ml of 20% sucrose solution in the bottom of each tube containing cell suspension.
11. Centrifuge the tubes at 700 rpm (100g) for 15 minutes at room temperature.
12. Discard upper layer; mix the deposit with 1 ml RPMI 1640.
13. Make nylon wool columns, flush with RPMI 1640 and incubate in moist box for 30 minutes at 37°C.
14. Pour 1 ml cell suspension in the column and incubate in the moist box for 30 minutes at 37°C.
15. For collection of T-cell, place the column upright in a test tube and pour 10 ml RPMI 1640 in the column. T-cells will be collected in the tube.
16. For collection of B-cells pour RPMI 1640 in the column and squeeze the nylon wool 2-3 times with a plunger. B-cells will be collected in the tube labelled ‘B’.
17. Centrifuge both tubes at 1800 rpm (525g) for 15 minutes.
18. Discard the supernatant; mix the deposit in each tube with 1 ml RPMI 1640. adjust the cell count at 2000 cells/µl.
19. Dispense 1 µl of T-cell suspension in each well of the tray containing HLA class I antisera.
20. Repeat the procedure for B-cell suspension. Dispense 1 µl cell suspension in each well of the tray containing HLA class II antisera.
21. Shake the trays and incubate class I (ABC) tray at room temperature for 30 minutes and class II (DR) tray at 37°C for 1 hour.
22. After incubation, add 5 µl rabbit complement to each well of both trays.
23. Shake the trays for mixing
24. Incubate again both trays at room temperature; class I (ABC) tray for I hour and class II (DR) tray for 2 hours.
25. Add 5 µl 5% eosin to each well.
26. Add 5 µl 40% formalin to each well after three minutes.
27. Apply cover slips and cover trays. Keep in refrigerator. Read after one hour.

Results and interpretation

Strength of reaction in each well is assessed on a scale of 0-8. HLA specificities are assigned according to the worksheet in every case.

Quality assurance

Commercially prepared negative and positive HLA controls are included in each tray.

Every new set is compared to the trays in use by testing the same individual on two trays.

32. SKIN TESTS

MANTOUX TEST

Mantoux test is the most commonly requested skin test performed by a clinical laboratory almost daily. The test is based on type IV hypersensitivity reaction. The positive reaction shows activation of memory T-cells generated in response to a previous exposure to Mycobacteria (see also TUBERCULIN SKIN TEST on page 148). Purified protein derivative (PPD) of *Mycobacterium tuberculosis* is used as antigen. PPD is standardised to specify number of tuberculin units (TU) in a known volume. One TU is the activity contained in a specified unit of internationally agreed standard PPD. Several strains of *Mycobacterium tuberculosis* can be used for preparation of PPD. These strains include PN, DT, C, & RT-23. WHO has recommended the use of RT-23 strain. It is usually available in single dose or multiple dose vials of 1, 5, 10, 100 & 250 TU contained in 0.1 ml volume.

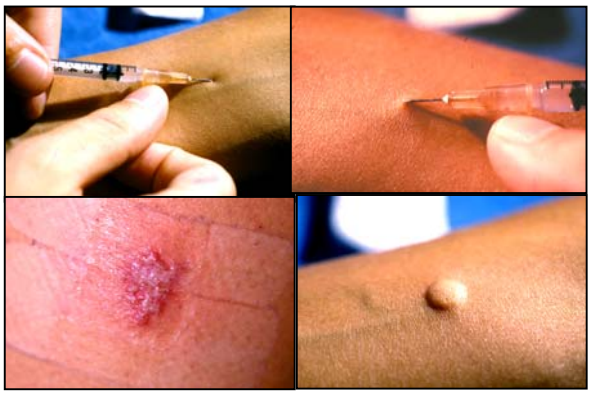


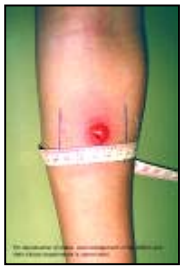
Figure 32.1: Technique of Mantoux test

Dose

The recommended testing dose is 5 TU, intradermally.

Technique

It is important to inject PPD **intradermally**. A subcutaneous injection does not elicit the standard response and the test may be read as negative. Skin of ventral surface of forearm is the site of choice. The best site is junction of upper and middle third of the forearm. Selected area should be free of visible veins. The test site should be sterilised with povidone iodine followed by alcohol swab. The skin is stretched and a fine needle is used. The needle is inserted in dermis to about 3 mm distance so that no amount of PPD leaks from the puncture site. If the needle is inserted properly, injection of even 0.1 ml of PPD will require considerable force and a bleb with pale surface will form (Figure 32.1). Once injected, the needle is gently withdrawn. A circle with non-washable ink is drawn around the injection site. The circle should have a diameter of 20 mm with injection site in the centre. Result is read after 72 hours. An immediate reaction to chemical contaminants may appear in the form of erythema but it subsides in 24 hours. True reaction is characterised by the appearance of induration (raised, red and tender lesion) after about 24 hours. It increases in size, attaining a peak in 72 hours and then gradually disappears. When pressed gently between finger and thumb, its thickness can be felt and tenderness is elicited.



Interpretation

Positive reaction: If an area of induration is observed, the reaction is reported according to the following protocol:

1. 5-10 mm induration is classified as:
   - Borderline positive. It is to be repeated after 8 weeks.
   - It is to be reported as positive in persons with fibrotic changes on chest radiograph consistent with old healed tuberculosis. May be considered positive in patients with organ transplant, immuno-suppressed or HIV-positive persons.
2. 10 mm induration is classified as positive.
3. 15 mm induration is classified as positive in persons with no known risk factors for tuberculosis (normal general population).

If the induration is observed in individuals with history of BCG vaccination, the result is interpreted according to the following protocol:

- It may reflect the booster phenomenon. *M.tuberculosis* infection (latent) is suspected if induration size is ≥10 mm, especially if vaccinated person is in contact with patient of infectious tuberculosis or is exposed continually to a population in which prevalence of tuberculosis is high.

- If an individual is HIV seropositive, induration of ≥5 mm is significant.
- If a person is immunocompromised and there is a history of contact with infectious patient, non-reactive tuberculin should be considered as infection with tuberculosis.

Negative reactions
If there is no reaction or the induration is <5 mm, the test is read as negative. A true negative Mantoux test implies that the person has never come in contact with living tubercle bacilli or the vaccine in the past.

False negative reaction
It may occur in following situations:
1. Anergy
2. Recent TB infection
3. Very young (<6 months old) or very old
4. Live-virus vaccination
5. Overwhelming TB disease like miliary tuberculosis
6. Infection with TB long time ago (two step test)
7. Chronic diseases (especially malignancies, end stage renal disease)
8. Poor nutrition
9. Poor skin elasticity (poor retention)
10. Viral infections e.g., Measles, Rubella etc.
11. Lymphomas
12. Sarcoidosis
13. T-Cell immunodeficiency diseases e.g., Wiskott-Aldrich syndrome, DiGeorge's anomaly, Nezelof's syndrome
14. Lepromatous leprosy
15. Intestinal lymphangiectasia
16. Chronic lymphocytic leukaemia
17. HIV infection
18. Recent BCG vaccination
19. Extreme debility
20. Defective tuberculin
21. Improper technique of injection

False positive reaction
It may occur in infection with non-tuberculosis mycobacteria and in cases with BCG vaccination.

Unwanted Reactions
This is seen as:
- Tuberculin shock: An injection of large dose of PPD to a highly sensitised subject may result in prostration, hypothermia and death.
- Fever
- Flare up of previously existing foci.
- Local inflammatory reaction at the site of inoculation.
- Constitutional symptoms: malaise, pain in limbs, vomiting, dyspnoea etc.

LEPROMIN TEST

This test is used to assess the immune response of an individual to *M. leprae*. This is similar to Mantoux test. Preparation of human lepromatous tissue is injected intradermally and the reaction is read after 48-72 hours. Reaction can be:
- **Positive**: It indicates good T-cell immunity.
- **Negative**: It is seen in cases without leprosy or with lepromatous leprosy.
- **Mistuda reaction**: This reaction develops after 3-4 weeks. Reaction can be read under microscope on biopsy. It indicates that the individual can react by a granulomatous response to the lepra bacilli. The test is positive in 90% of normal persons and indicates good immunity. Such persons are unlikely to develop leprosy.

FREI TEST

This is an intradermal test used in the diagnosis of lymphogranuloma venereum. The egg yolk grown *Chlamydia trachomatis* (L1-L3) antigen is heat inactivated and 0.1 ml is injected intradermally. The reaction is read after 48-72 hours. An induration of 6 mm diameter constitutes a positive test. Cross-reactions with other *chlamydiae* may occur. The Frei antigen is genus specific so this test lacks specificity. Moreover, the test lacks sensitivity in the early stages of the disease. This test is rarely used these days.

FUNGAL SKIN TESTS

Fungal antigens are injected intradermally. The reaction is read as in the case of Mantoux test. These tests are used to diagnose systemic diseases due to fungi.

SCHICK TEST

This test was in use to determine the immune status of an individual against diphtheria organisms (see also Schick test on page 133). It is now obsolete and is given here for historical interest. The basis of the test is that if diphtheria toxin is injected intradermally, it is very irritating and results in local damage of subcutaneous tissue. If antitoxin against the diphtheria bacilli is present in an individual, the injected toxin is neutralised and there is no tissue damage. It is a good example of neutralisation test. The toxin is used in a dose that is equal to 0.001 unit of US standard diphtheria antitoxin. The toxin is injected into the forearm of the person. The other arm is injected with a control, which is a

heat-inactivated toxin. The test is read after 24 hours, 48 hours and 6 days.

Interpretation

Following results can be observed:

Positive test: Toxin produces redness and swelling in the test arm that increases for several days and then fades. The control arm gives no reaction. A positive test indicates susceptibility to diphtheria as the individual has inadequate levels of antitoxins in his body. These individuals need to be immunised against diphtheria.

Negative test: No reaction on either arm. This means that the individual has sufficient antitoxin in the body to protect him from the injurious effects of the toxin. These individuals do not need immunisation against diphtheria.

False positive test: Redness appears on both arms within 24 hours. It fades on both arms in 2 or 3 days. This indicates hypersensitivity to components in the toxin other than diphtheria toxin. Such individuals are susceptible to diphtheria but cannot be immunised, as they are hypersensitive to the toxin. Immunisation is contraindicated in such cases.

Combined reaction: This reaction begins with redness and swelling on both arms in 24 hours. The redness and swelling from the control arm disappears on 2nd and 3rd day. The reaction on the test arm clears in several days as that of positive reaction. This shows both hypersensitivity and immunity. No immunisation for diphtheria is required.

SKIN PRICK TEST

Indications: The principal indication for skin prick testing is a reasonable suspicion that a specific allergen or group of allergens is triggering symptoms of rhinitis, conjunctivitis, or asthma in an allergic patient.

Precautions: Several precautions should be observed during any form of skin testing.

1. Withhold antihistamine drugs at least 72 hours before the test. Inhalers and steroids can be continued.
2. Testing should not be done during periods of symptomatic bronchospasm, to prevent potential worsening of the clinical state.
3. Emergency treatment materials, syringes and needles should be readily available.

Method: Skin prick testing can be performed on any flat skin surface but forearm or the back are the conventional sites. If the forearm is used, avoid the skin is the antecubital fossa or near the wrist.

1. Clean the skin with methylated spirit and allow it to dry by evaporation.
2. Using a skin-marking pen, mark out and number skin test sites at least 2 cm apart to prevent reagent mixing/ positive reaction coalescing.
3. Record skin test antigens to be used and check that numbering conforms to marked skin sites.
4. Ensure adequate mixing of the skin test solutions; then place one drop of allergen extract on the skin at the appropriate point. Using a fresh disposable 26-guage needle, prick carefully the superficial layer of the skin with a lifting motion.
5. **Do not draw blood.**
6. Observe the patient throughout the test till reactions are read. Instruct the patient to inform instantly about any feeling of heart sinking, sweating, palpitation etc.
7. Reactions are read after 15 min. A positive reaction is suggested by itching within a few minutes and confirmed by the typical palpable weal with surrounding erythema. The average (greatest and smallest, at right angles) diameter of the weal in millimeters is recorded and compared to negative controls.

Control: A negative control of diluent solution alone is also included to assess skin reactivity to mechanical trauma (for instance, in patients with dermographism).

Interpretation and Limitations

1. A positive skin test result can be present in a symptomless subject.
2. A positive skin test in a symptomatic person is usually significant when correlated with the clinical history, but a skin test can remain positive for years after cessation of symptoms.
3. Some patients have target organ sensitivity but lack skin sensitivity to the antigen.
4. Differences in the stability and purity of extracts also affect biological potency, and the preservative used in older preparations to improve stability and prevent contamination (e.g. phenol) can have non-specific irritant effects.
5. The magnitude and reproducibility of the response is often influenced by biological variability; skin reactivity being greatest at about the third decade but declining from the fifth decade onwards. False-negative results may occur in the very old, in infants and in toddlers.
6. Skin reactivity may vary with circadian rhythms and menstrual cycles and, in the

presence of dermographism, there will be positive skin tests to all antigens, including negative saline controls.

7. Since antihistamines (H1 receptor antagonists) suppress skin test reactivity, they should be discontinued at least 72 hours prior to testing and preferably for 5 days. Newer long-acting antihistamines, such as astemizole, should be stopped for at least 8 weeks before testing. Oral beta-2 agonists, sodium cromoglycate and corticosteroids do not interfere with immediate skin reactions.
8. Prick tests can be helpful in patients with moderate or high degrees of sensitisation to inhaled antigens; overall, skin tests and provocation tests agree in about three-quarters of patients.
9. Properly used, positive skin tests help to distinguish allergic rhinitis from non-allergic causes, such as vasomotor rhinitis.

SECTION VI – HAEMATOLOGY

33. THEORETICAL ASPECTS

HAEMOPOIESIS

The blood consists of a fluid part called plasma and the formed elements called cells. The blood cells are of three types, red blood cells (RBC), white blood cells (WBC) and platelets (Plt). White blood cells are further divided into three main groups, granulocytes (neutrophils, eosinophils and basophils), monocytes and lymphocytes. The blood cells are continuously destroyed either by aging or as a result of their functional activities and are replaced by new cells. There is a fine balance between the rates of formation and destruction of these cells in healthy people. Formation of blood cells is termed haemopoiesis.

SITES OF BLOOD FORMATION

In first 19-20 days of embryonic stage, blood cells are formed in the wall of the yolk sac in blood islands. These cells are mesodermal in origin; hence this phase of haemopoiesis is called Mesoblastic Phase. **Mesoblastic haemopoiesis** produces only RBC, which remain nucleated throughout their life span. The haemoglobin in these RBC is also most primitive, called embryonic haemoglobin. The liver is the main site of haemopoiesis in the foetus from 5th to 30th week of intra-uterine life. Some haemopoiesis continues in the liver even after birth for 1-2 weeks. This is termed **Hepatic Phase of haemopoiesis**. All types of blood cells are produced in the later part of this phase. RBCs produced in this phase are larger than the adult RBC but are non-nucleated. These contain less primitive haemoglobin called foetal haemoglobin. The **bone marrow** gradually takes over haemopoietic function from the fifth month until term when it is the only major site for formation of blood cells. Lymphocyte precursors are formed in the liver and bone marrow but the main sites for lymphocyte production are spleen, lymph nodes and other lymphoid tissue. Initially haemopoiesis takes place in the marrow of all bones but after birth it slowly and gradually recedes to marrow of flat bones and vertebrae. At birth bone marrow constitutes 1.5% of body weight, which increases to 4.5% in the adult age. However in children 75% of the total marrow is haemopoietic whereas in the old age only 30-40% of the marrow is haemopoietic. In young adults 50% of the total marrow is haemopoietic. Non-haemopoietic marrow, at all ages, consists of fat cells.

Gestational age	Phase of haemopoiesis	Location
2 weeks - 2 months	Mesoblastic: Begins in yolk sac wall where small nest of blood cell production can be seen, referred to as blood islands	Wall of yolk sac
6 weeks - birth	Hepatic: Islands of blood cell development occur within liver parenchyma. Dominant site for first half of gestation, also occurs to some extent within spleen	Liver
2.5 months-birth	Myeloid: Within bone marrow, begins in clavicle at 2.5 months, continues to rise until myeloid tissue becomes major site of haemopoiesis in latter half of gestation.	Bone marrow

ORIGIN OF BLOOD CELLS

All blood cells are formed from the undifferentiated primitive cell, which resembles a large lymphocyte and is called **pleuripotent** or **totipotent haemopoietic stem cell**. It gives rise to **lineage specific stem cells**, termed colony forming units lymphoid and spleen (CFU-L & CFU-S). These in turn differentiate into more **committed stem cells** and **progenitor cells** that can only differentiate on specific lines. These are also called CFUs and include CFU-T (for T-lymphocytes), CFU-B (for B-lymphocytes), CFU-GM (for granulocytes and monocytes), CFU-Eo (for eosinophils), CFU-Meg (for megakaryocytes), **Burst Forming Units** for Erythroid cells (BFU-E) and CFU-E. Rests of the pathways are shown in Figure 33.1.

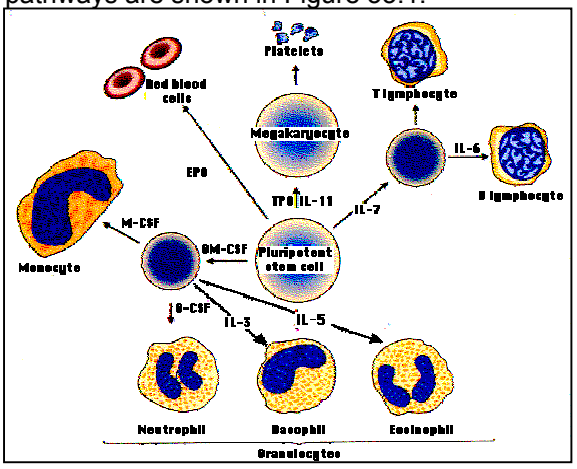


Figure 33.1: Schematic representation of Haemopoiesis

The stem cells maintain their number by **self-renewal**. When the need arises, a stem cell divides into two. One of the daughter cells replaces the parent cell in stem cell pool while the other differentiates along the required cell line. All

of this takes place under the influence of certain proteins, which are called **haemopoietic growth factors**. These include **interleukins** (IL) and colony stimulating factors (CSF), which are secreted by various cells in response to stimuli. Important haemopoietic growth factors include IL-3, GM-CSF, G-CSF and **Erythropoietin** (Epo). There are certain other proteins that have an inhibitory influence on haemopoiesis. For example, Interferon (INF) and Tumour Necrosis Factor (TNF).

STEPS IN BLOOD FORMATION

Formation of each type of blood cell is named after the cell line. Formation of RBC is called **Erythropoiesis**, formation of granulocytes is called **Granulopoiesis**, formation of platelets is called **Thrombopoiesis** and formation of lymphocytes is called **Lymphopoiesis**. Formation of blood cells and their delivery into the blood stream involves three processes.

1. **Multiplication/Proliferation**, which takes place by successive division of stem and progenitor, cells by the process of mitosis.
2. **Maturation/Differentiation** that occurs by progressive development of specific structural and functional cell-characteristics.
3. **Release of mature cells** from the marrow into the blood stream. Some maturation normally occurs after release of cells e.g., maturation of reticulocytes to RBC. Immature forms may be released into circulation under conditions of stress

ERYTHROPOIESIS

In normal marrow the proerythroblast is the first identifiable cell of the erythroid series. It divides and matures to a RBC through various stages. The process of normoblastic maturation is characterised by the following progressive changes.

- Decrease in cell size
- Haemoglobinisation
- Extrusion of the nucleus.

The time for maturation from pronormoblast to mature red cell is about 7 days. Various stages in development of a RBC are (Figure 33.2):

1. **Pronormoblast**: It is a round cell with a diameter of 12-20 µm. It has a large nucleus surrounded by a small amount of cytoplasm. The cytoplasm is deep blue in colour. The nucleus is round and consists of a network of uniformly distributed chromatin strands. It is reddish purple in colour and contains several nucleoli (Plate-I). It divides and matures to basophilic or early normoblast.
2. **Basophilic (Early) normoblast**: It is 10-16 µm in diameter. It has a large nucleus with thick chromatin strands and no nucleoli. Cytoplasm is blue like the pronormoblast. It divides and matures into polychromatic or intermediate normoblast.
3. **Polychromatic (Intermediate) normoblast**: It is 8-14 µm in diameter. The nucleus occupies a smaller part of the cell and stains deeply. The cytoplasm gives a reddish tinge and is not so blue in colour due to the formation of haemoglobin. It divides and matures into Orthochromatic or late normoblast.
4. **Orthochromatic (Late) normoblast**: It varies from 8 to 10 µm in diameter. The cytoplasm is acidophilic (red) due to haemoglobinisation. The nucleus is small and appears as deeply staining blue-black homogeneous mass (pyknotic). It becomes eccentric in position and is finally extruded out from the cell. Late normoblast cannot divide and only matures into reticulocyte by extrusion of nucleus.
5. **Reticulocyte**: The reticulocyte is a flat disc-shaped cell. It has no nucleus and is slightly larger than the mature red cell. It has diffuse basophilic (bluish) tinge (polychromatic). With supravital stains such as brilliant cresyl blue, the basophilic material, which is RNA, appears in the form of a reticulum. The reticulocyte becomes a mature red cell in about 1-4 days. Half of this time is spent in spleen.

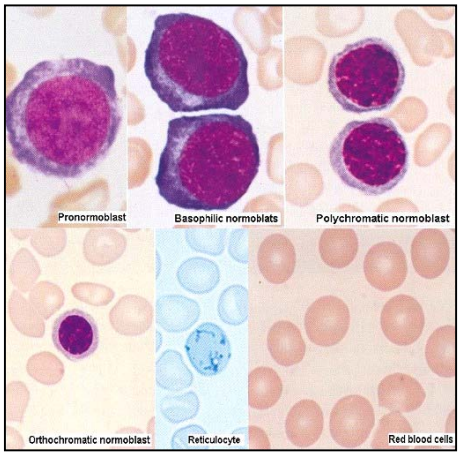


Figure 33.2: Erythropoiesis

6. **Red Blood Cell (RBC)**: The mature RBC is a non-nucleated cell. It is a biconcave disc. It is about 7.2 µm in diameter. The cytoplasm is pink due to the presence of haemoglobin. There is no nucleus, no mitochondria and no ribosomes (see also page 240).

GRANULOPOIESIS

The earliest recognisable cell of granulocytic series in the bone marrow is myeloblast. It divides

and matures into various granulocytes in stages. The process is characterised by:

- Change in the size of the cell
- Maturation and lobulation of the nucleus
- Production of specific granules in the cytoplasm.

The time for maturation from myeloblast to mature granulocyte is about 4 days. Various stages in development of a granulocyte are (Figure 33.3):

1. **Myeloblast**: It is the first recognisable cell of this series. It has a large round or oval nucleus, which occupies most of the cell and contains 2-4 nucleoli. The cytoplasm is non-granular and deep blue in colour. It divides and matures into Promyelocyte.
2. **Promyelocyte**: It is the next cell in the white cell series. It resembles myeloblast, but is larger, has more cytoplasm, which contains purplish red granules (azurophilic granules). The nucleus still contains some nucleoli or their remnants. It divides and matures into Myelocyte.
3. **Myelocyte**: The next stage in granulopoiesis is myelocyte, which differs from the promyelocyte in two respects. First, the cytoplasmic granules develop their specific character (purplish for neutrophils, eosinophilic for eosinophils, basophilic for basophils). Second the nucleus has no nucleoli. The diameter of myelocyte may be up to 25 µm. The cytoplasm is light blue in the early stages and acquires pinkish colour with maturation. The nucleus is round or oval and contains no nucleoli. Myelocyte does not divide and only matures into a metamyelocyte.
4. **Metamyelocyte**: The nucleus of this cell is small, eccentric and slightly indented. The cytoplasm is pinkish and contains specific granules. This cell is slightly smaller in size than the myelocyte. The specific granules are more abundant.
5. **Band (Stab) form**: It is a mature metamyelocyte, which has a band like nucleus adapted to a U shape. The specific granules are abundant.
6. **Mature Granulocyte**: Depending upon the type of specific granules these are of three types:
   a. **Neutrophil**: It is 12-14 µm in diameter. The nucleus is lobulated having two to five lobes that are connected by thin chromatin strands. The cytoplasm is pink and contains numerous fine purplish granules.
   b. **Eosinophil**: The mature eosinophil is slightly larger than the mature neutrophil. Its average diameter is about 16 µm. The nucleus usually has two lobes. The cytoplasm is packed with relatively larger granules, which do not overlap the nucleus. The granules stain reddish orange with Romanowsky stains.
   c. **Basophil**: The mature basophil has a lighter staining nucleus than the neutrophil. It seldom contains more than two lobes. The cytoplasm is pink and contains a number of large oval or round, deeply staining basophilic granules. They do not pack the cytoplasm as do eosinophilic granules but overlie the nucleus.

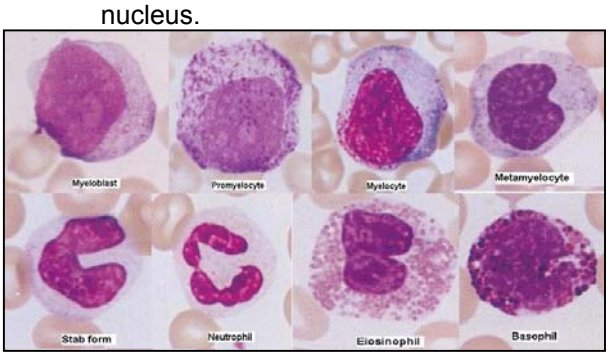


Figure 33.3: Myelopoiesis

MONOPOIESIS

Monocytes are formed mainly in the bone marrow and migrate to the spleen, lymphoid and other tissues and organs of the body where these are transformed into macrophages. Various stages in its development are:

1. **Monoblast**: It is the earliest recognisable cell of the series. It is a large cell similar in structure to the myeloblast. Nuclear outline is, however, not as regular as in myeloblast and may show indentation or convolution.
2. **Promonocyte**: It is a large cell about 20 µm in diameter. It has abundant cytoplasm, grey blue in colour and may contain fine azurophilic granules. The nucleus is usually round or kidney shaped giving folded appearance but may be lobulated.

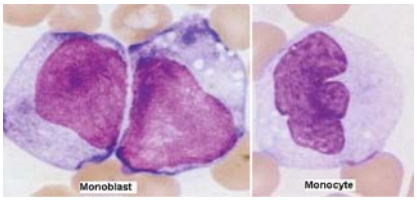


Figure 33.4: Monopoiesis

3. **Monocyte**: It is slightly smaller than promonocyte. Other features are similar. Its cytoplasm has typical ground glass appearance. The nucleus is like a band folded upon itself to assume a spherical shape.

LYMPHOPOIESIS

Mature lymphocytes develop mainly in the lymphoid tissues of the body, namely lymph nodes, spleen, gastrointestinal tract and tonsils. Bone marrow makes only a small contribution to lymphocyte production. CFU-L probably migrates to lymphoid tissue early in life. These also develop through stages (Plate-III). The maturation of lymphocytes is characterised by:

- Maturation of nucleus and cytoplasm
- Adaptation to their function by expression of specific proteins.

1. **Lymphoblast**: It is the earliest recognisable cell of the series. It measures 15-20 µm in diameter and contains a large, round or oval nucleus. Nucleoli are present, usually 1-2 in number. The cytoplasm is non-granular and deep blue in colour forming a narrow rim around the nucleus.
2. **Prolymphocyte**: It is the next stage in formation of lymphocyte. Nucleus contains a prominent nucleolus, usually centrally placed. Cytoplasm is variable.
3. **Large lymphocyte**: It is about 12-16 µm in diameter. Cytoplasm is sky blue in colour and contains few granules, which stain purplish red. The nucleus is round or slightly indented. Nucleoli are absent.
4. **Small lymphocyte**: The large lymphocyte matures into small lymphocyte. It is 9-12 µm in diameter. The cytoplasm is scanty and stains blue. Purplish red granules may be present. The nucleus is round or slightly indented. Nucleoli are absent.

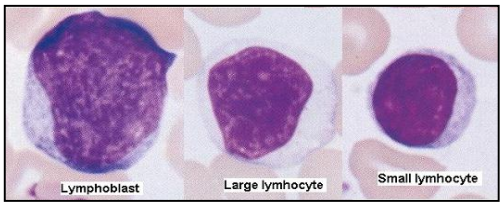


Figure 33.5: Lymphopoiesis

THROMBOPOIESIS

Platelets are formed from the cytoplasm of a large cell in the bone marrow known as megakaryocyte. This also passes through various stages of development in the bone marrow. These are:

1. **Megakaryoblast**: It is a large cell about 20-30 µm in diameter. It has a large oval or indented nucleus that contains several nucleoli. The cytoplasm is blue, small in amount and contains no granules. It may show budding.
2. **Promegakaryocyte**: It is formed from the megakaryoblast. It is larger than the megakaryoblast. It has deep blue cytoplasm that contains azurophilic granules. The nucleus is non-lobulated or partly lobulated. From here onward only the nucleus divides while the cell enlarges without division (Endomitosis).
3. **Megakaryocyte**: It is a large cell, from 30-90 µm in diameter. It contains a single multi-lobulated or indented nucleus. The number of nuclear lobes varies from 4-16 depending upon the number of divisions it has undergone. The cytoplasm is abundant and stains light blue. It contains fine azurophilic granules. The margin is irregular and may show pseudopod formation.

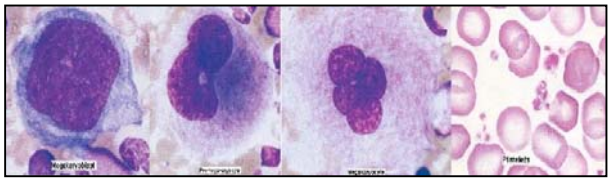


Figure 33.6: Thrombopoiesis

4. **Platelet**: It is a small discoid structure, 1-2 µm in size. These are formed by partitioning of cytoplasm of megakaryocyte into numerous structures that separate to form platelets.

ANAEMIAS

Anaemia is defined as a decrease in haemoglobin level (or total circulating red cell mass) for the age and sex of a person. The influence of sex is important after puberty. The haemoglobin level in adult females is lower as compared to adult males of the same age group. This is because of the influence of menstrual loss and lack of androgens. Haemoglobin (Hb) is contained in RBCs, which circulate in blood. These are biconcave discs, 6.7-7.7 (mean 7.2) µm in diameter. Their number in circulation of an adult male normally is 4.5-5.5 (mean 5.0) $X10^{12}$/L. Each RBC has a volume of 92 fl and contains 29.5 pg of Hb, the concentration of which in an individual RBC is 33 g/dl. Normal life span of a RBC, in peripheral blood is about 120 days (see also page 238).

CLASSIFICATION AND AETIOLOGY

There are various criteria for classification of anaemia. Each type of classification has certain advantages and disadvantages. For routine laboratory work, the morphological classification is most useful. In this classification anaemias are divided into three main groups depending upon the size of RBC and amount of haemoglobin present in each cell. These groups can be identified by measuring absolute values as well as by examination of red cell morphology on stained blood film. These groups are:

1. **Microcytic Hypochromic Anaemia**: In this type of anaemia individual RBCs are smaller in size than normal and contain subnormal amount of haemoglobin. All absolute values (MCV, MCH, and MCHC) are below normal. This type of anaemia is commonly seen in:
   - Iron deficiency
   - Thalassaemia
   - Sideroblastic anaemia
   - Anaemia of chronic disorders (some cases)
2. **Macrocytic Anaemia**: In this type of anaemia individual RBCs are larger than normal, the amount of haemoglobin in each cell is usually below normal. Absolute values show increased MCV with usually normal MCH/MCHC. This type of anaemia is commonly seen in:
   - Megaloblastic anaemia
   - Aplastic anaemia
   - Haemolytic anaemia
   - Liver disease
   - Myxoedema
   - Hypopituitarism
   - Pregnancy
   - Alcoholism
3. **Normocytic Normochromic Anaemia**: In this type of anaemia, although the haemoglobin concentration in blood is reduced the individual RBCs appear normal and absolute values are also within normal limits. This type of anaemia is seen in:
   - Acute blood loss
   - Leukaemia
   - Bone marrow infiltration
   - Chronic renal failure
   - Chronic infections (Chronic disorders)

DIAGNOSIS

Following investigations are to be performed for diagnosing a case of anaemia:

- Estimation of Haemoglobin (Hb).
- Estimation of Total Red Blood Cell Count (TRBC).
- Estimation of Haematocrit (Hct) or Packed Cell Volume (PCV).
- Calculation of absolute values.
- Examination of peripheral blood film.
- Reticulocyte count

After determining the morphological type of anaemia, the patient is further investigated to determine the cause of it (see also the section on Biochemical Investigations of Anaemia on page 344).

HAEMATOLOGICAL MALIGNANCIES

The haematological malignancies arise from uncontrolled clonal proliferation of the cells of haemopoietic system. These include:

- Leukaemias
- Lymphomas
- Myeloproliferative disorders
- Myelodysplastic syndromes
- Plasma cell dyscrasias
- Malignant disorders of monocyte macrophage system

LEUKAEMIAS

Leukaemia can be defined as malignant proliferation, abnormal maturation and accumulation of various cells in the hierarchy of haemopoietic cells. These can be divided into acute and chronic leukaemias based on clinical course of the disease and state of maturation of the malignant cells in blood and bone marrow.

Acute Leukaemias

Acute leukaemias usually have a rapid onset and are characterised by the presence of 20% or more blast cells in the bone marrow. The acute leukaemias have been classified by a group of French, American and British haematologists into various groups and sub-groups with well defined morphological and cytochemical criteria (FAB classification). The two main groups are acute myeloid leukaemias (AML) and acute lymphoblastic leukaemias (ALL).

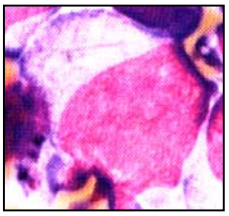


Figure 33.7: Myeloblast fwith Auer rod

Acute Myeloid Leukaemia: The acute myeloid leukaemias, some times called acute non-lymphoblastic leukaemias (ANLL), are subdivided into 8 sub-groups M0 to M7. The original FAB classification is based on morphology of blasts in the bone marrow stained with Romanowsky stains and Sudan Black-B (SBB) or myeloperoxidase (MPO) stains except in case of AML M0 where an anti-myeloperoxidase antibody is used to demonstrate MPO in the blast cells. Salient features of this classification are as follows:

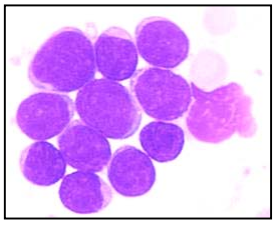


Figure 33.8: Acute myeloid leukaemia (AML-M0)

AML-M0 (Acute Myeloid Leukaemia without MPO expression): It is characterised by the presence of Type-I blasts. These react positively with anti-

MPO antibody. Blasts constitute 20% or more of all nucleated cells in the bone marrow.

AML-M1 (Acute Myeloid Leukaemia without maturation): This type of AML is characterised by the presence of type-I and type-II blasts which constitute 20% or more of all nucleated cells in the bone marrow but more than 90% of non-erythroid cells. Occasional cell shows Auer rod (Figure 33.7). Three percent or more of blasts are SBB/MPO positive.

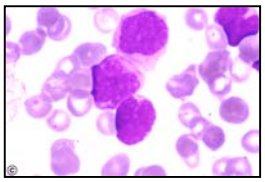


Figure 33.9: Acute myeloid leukaemia (AML-M1)

AML-M2 (Acute Myeloid Leukaemia with maturation): This type of AML is similar to MI with two exceptions. First, the blasts constitute less than 90% of non-erythroid cells in the bone marrow and second that monocytic component in the bone marrow is less than 20%. Auer rods are more frequent.

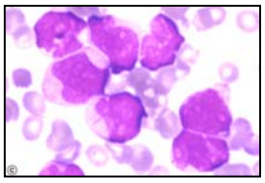


Figure 33.10: Acute myeloid leukaemia (AML-M2)

AML-M3 (Acute Promyelocytic Leukaemia): This type of AML is characterised by accumulation of abnormal promyelocytes, some times called type-III blasts, in the bone marrow. These are large, heavily granulated promyelocytes with multiple Auer rods. In some cells these Auer rods are so numerous that they form a mass called faggot body. These stain intensely positive with SBB/MPO. In a variant M3 the granules, Auer rods and faggot bodies are scanty.

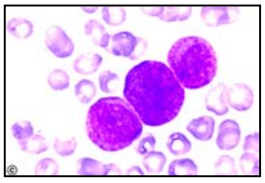


Figure 33.11: Acute promyelocytic leukaemia (AML-M3)

AML-M4 (Acute Myelomonocytic Leukaemia): This type of AML also takes into account one feature in peripheral blood as well i.e. absolute monocyte count, which should be more than $1x10^9$/L. In the bone marrow the blasts constitute more than 30% of non-erythroid cells and monocytic component is more than 20%.

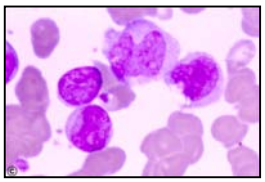


Figure 33.12: Acute myelomonocytic leukaemia (AML-M4)

AML-M5 (Acute Monocytic Leukaemia): Acute monocytic leukaemia is characterised by the presence of more than 30% type-I blasts of non-erythroid cells in the bone marrow but total monocytic component (monoblasts, promonocytes and monocytes) constitute more than 80%. The blasts are larger, nucleus is irregular, sometimes giving a convoluted appearance and cytoplasm has ground glass appearance.

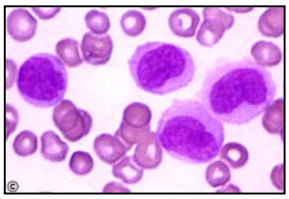


Figure 33.13: Acute monocytic leukaemia (AML-M5)

AML-M6 (Acute Erythroblastic Leukaemia): In this category erythroid cells constitute more than 50% of all nucleated cells in the bone marrow. These have megaloblastic features i.e. these are larger than normal erythroblasts and have open chromatin. Some-times these are binucleate or even multinucleate (gigantoblasts). These cells show large Periodic Acid Schiff (PAS) positive granules. There are also present type-I or type-II blasts, which constitute more than 30% of the non-erythroid cells.

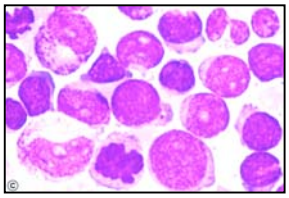


Figure 33.14: Acuteerythroblastic leukaemia (AML-M6)

AML-M7 (Acute Megakaryoblastic Leukaemia): The blasts, in this type of AML, constitute more than 30% of all cells in the bone marrow and are predominantly of type-I. Some blasts show budding of the cytoplasm into platelet like structures, which stain positively with PAS stain.

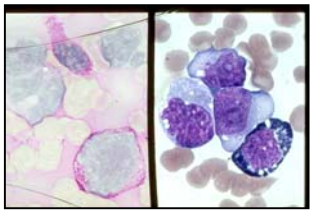


Figure 33.15: Acute megakaryoblastic leukaemia (AML-M7)

Acute Lymphoblastic Leukaemias: Acute lymphoblastic leukaemias are divided into three sub-groups by the FAB group, based on morphology of blasts in the bone marrow stained with Romanowsky stains. These types are ALL-L1, ALL-L2 and ALL-L3. The salient features of these sub-groups are as under.

ALL-L1: In this type of ALL the blasts are small in size with scanty cytoplasm. Nucleus is mostly regular in shape with occasional cell showing cleft or indentation of nucleus. The chromatin is homogenous and nucleoli are inconspicuous.

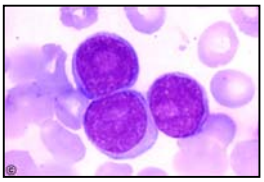


Figure 33.16: ALL-L1

ALL-L2: In this type blasts are of heterogeneous

size but predominantly large. Nuclear shape is predominantly irregular, showing frequent clefts or indentation. Nuclear chromatin is heterogeneous and nucleoli are large and prominent. Many times it is difficult to differentiate between ALL-L1 and ALL-L2. To overcome this problem a scoring criteria has been suggested. This is outlined in Table 33.1.

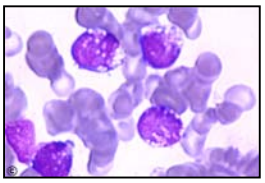


Figure 33.17: ALL-L2

ALL-L3: Morphologically it is the most distinct sub-group of ALL. Blasts are large but heterogeneous. Nuclei are regular and oval to round in shape. Nuclear chromatin is homogenous and finely stippled. Nucleoli are prominent and vesicular. The cytoplasm is relatively abundant, deeply basophilic and contains several vacuoles in the cytoplasm.

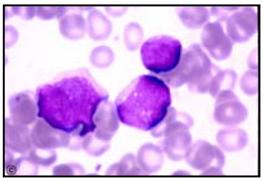


Figure 33.18: ALL-L3

Table 33.1: Scoring system for ALL

Cell Character	Score
High nucleocytoplasmic ratio in at least 75% of cells	+1
Low nucleocytoplasmic ratio in at least 25% of cells	-1
No more than one and inconspicuous nucleolus in at least 75% of cells	+1
One or more prominent nucleoli in at least 25% of cells	-1
Irregular nuclear out line in at least 25% of cells	-1
At least 50% cells are large (twice a normal small lymphocyte)	-1

Score 0 to +2 = ALL-L1
Score –1 to –2 = ALL-L2

Recently the immunological classification of ALL has gained popularity because of its correlation with prognosis of the disease. The classification is based on demonstration of lineage specific antigens in the cytoplasm or on the cell membrane of the blasts. This classification recognises Precursor Cell ALL, Pre-B ALL, Common ALL and T-ALL besides some hybrid groups.

Chronic Leukaemias: Chronic Leukaemias are characterised by chronic course of the disease and mature nature of the malignant cells. These include:

- Chronic granulocytic/myeloid leukaemia (CGL/CML).
- Chronic lymphocytic leukaemia (CLL)
- Chronic myelomonocytic leukaemia (CMML)
- Hairy cell leukaemia (HCL)

Of these CGL is also classified with myeloproliferative disorders but it is more appropriate to consider it under chronic leukaemias. Similarly CMML is also classified under Myelodysplastic Syndromes, which is more appropriate.

1. Chronic Granulocytic/Myeloid Leukaemia: CGL is characterised by chronic course, splenomegaly and high total leucocyte count in peripheral blood. Differential leucocyte count shows all stages, blast to mature granulocyte, of all types of granulocytes.

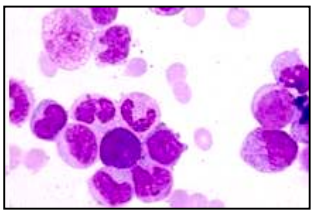


Figure 33.19: Chronic granulocytic/myeloid leukaemia

Basophils are usually increased. There is **bi-modal peak** that is myelocytes and mature forms are more abundant whereas metamyelocytes are less in number. Being abnormal cells these cells have very low activity of normal enzymes e.g., leucocyte/neutrophil alkaline phosphatase (LAP/NAP). A scoring system based on NAP staining has been evolved to differentiate between leukemoid reaction and CGL. Philadelphia chromosome, t(9;22), can be demonstrated in about 90% of cases whereas bcr/abl hybrid gene can be demonstrated in almost 100% of cases.

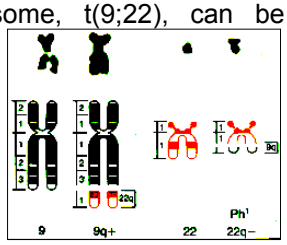


Figure 33.20: Pfhiladelphia chromosome

CGL has three phases; each characterised by particular clinical and laboratory features. These are **chronic phase**, accelerated phase and blast transformation. Almost every patient, if not treated with curative therapy, eventually develops blast transformation when the leukaemia becomes acute. The **accelerated phase** is characterised by worsening of clinical condition with development of anaemia and thrombocytopenia with or without increase in basophils to 20% or more. The blast count both in peripheral blood and bone marrow increases but it does not exceed in the marrow beyond 30%. Fibrosis may increase in the bone marrow and nucleated RBCs appear in peripheral blood. **Blast transformation** or crisis is characterised by presence of more than 30% blasts in the bone marrow in addition to features described for accelerated phase. Both myeloid and lymphoid blast transformations may occur but later is less common (one-third cases).

2. Chronic Lymphocytic Leukaemia: CLL is characterised by chronic course, splenomegaly and/or lymphadenopathy and high total leucocyte count in peripheral blood. It is further sub-classified into CLL proper prolymphocytic leukaemia (PLL) and a mixture of the two (CLL/PLL) based on stage of maturation of majority of malignant cells. Three stages of the disease have been recognised, based on clinical and laboratory features. This is called **Binet staging** and is important from management point of view. This system takes into consideration Hb concentration, platelet count and number of lymphoid areas involved. Five areas of lymphoid tissue are considered. These are lymph nodes of head and neck, lymph nodes of axilla, lymph nodes of groin, spleen and liver. In **stage A,** Hb is more than 10g/dl, platelet count is more than $100x10^9$/L and less than three lymphoid areas are involved. In **stage B**, Hb and platelet are same but more than 3 lymphoid areas are involved. In **stage C** any number of lymphoid areas may be involved but either the Hb is less than 10g/dl or platelet count is less than $100x10^9$/L or both.

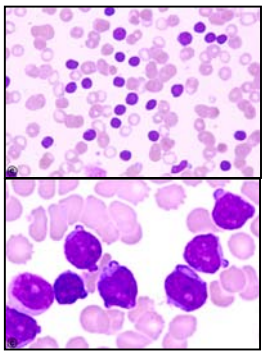


Figure 33.21: Chronic lymphocytic leukaemia. Low power (above), High power (below)

3. Hairy Cell Leukaemia: Hairy cell leukaemia (HCL) is characterised by old age, massive splenomegaly, pancytopenia in peripheral blood and presence of hairy cells in peripheral blood and bone marrow. Hairy cells are of the size of a large lymphocyte with conspicuous nucleolus and the cytoplasm drawn out into hair like processes. These cells stain positively for acid phosphatase, which is resistant to tartrate (TRAP).

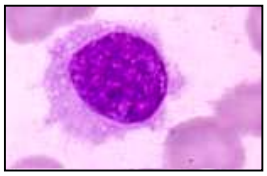
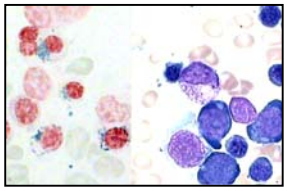


Figure 33.22: Hairy cell

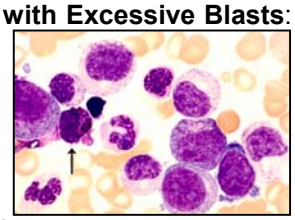
MYELODYSPLASTIC SYNDROMES

Myelodysplastic Syndromes (MDS) are a set of conditions that finally evolve to AML and are hence considered to be preleukaemic. These are characterised by no organomegaly, pancytopenia in peripheral blood and hypercellular marrow with dysplastic features with or without increased number of blasts (between 5-30%) or abnormal sideroblasts in increased number. MDS has been classified into the following groups by the FAB group:

1. **Refractory anaemia**: The bone marrow shows erythroid hyperplasia and/or dyserythropoiesis manifested by low reticulocyte count in peripheral blood.
2. **Refractory Anaemia with Ring Sideroblasts**: Ring sideroblast is defined as an erythroblast with a ring of more than 6 siderotic granules around the nucleus. RARS is characterised by features seen in RA together with sideroblasts constituting at least 15% of the erythroid cells.



3. **Refractory Anaemia with Excessive Blasts**: In addition to dysplastic features the bone marrow shows more than 5% but not more than 20% blasts and no blast has any Auer rod.



4. **Refractory Anaemia with Excessive Blasts in Transformation**: In addition to features of RAEB the blasts are more than 20% but not more than 30% and/or show Auer rods.
5. **Chronic Myelomonocytic Leukaemia**: This condition has the features of RAEB together with more than $1x10^9$/L monocytes in peripheral blood. Peripheral blood count is usually high and shows features of CGL. NAP score is not low.

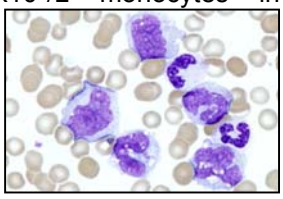


Figure 33.23: Chronic myelomonocytic leukaemia

MYELOPROLIFERATIVE DISORDERS

These disorders are characterised by uncontrolled proliferation of myeloid progenitors in haemopoietic stem cell hierarchy with accumulation of mature cells of the series. These disorders ultimately may transform to Acute Leukaemia. These include:

1. **Polycythemia Rubra Vera (PRV)**: In this disorders mature RBC are increased with increase in absolute red cell mass.
2. **Chronic myeloid leukaemia (CML)**: In this disorder mature elements of granulocytic cell series accumulate. This has been discussed in

detail under chronic leukaemias.

3. **Essential thrombocythemia (ET)**: In this disorder there is increase in absolute number of platelets.
4. **Primary myelofibrosis**: In this disorder, instead of proliferation of haemopoietic cells, there is marked proliferation of fibroblasts in the bone marrow with increased reticulin formation and collagenisation. This results in extra medullary haemo-poiesis manifesting with leucoerythroblastic blood picture, tear drop cells and extensive fibrosis in the bone marrow trephine biopsy.

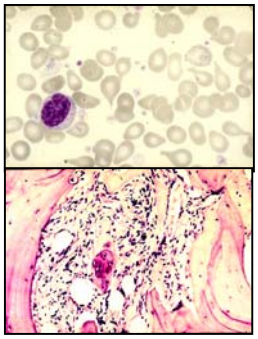


Figure 33.24: Tear drop cell (above), bone marrow fibrosis (below)

MALIGNANT DISORDERS OF MONOCYTE MACROPHAGE SYSTEM

In this group there is uncontrolled proliferation and accumulation of histiocytes. These include malignant histiocytosis of various types. The disorders are not very common and their description is beyond the scope of this manual.

LYMPHOMAS

Lymphomas are malignant neoplasms of lymphoid tissue. These are broadly divided into Hodgkin's and Non-Hodgkin's lymphomas. **Hodgkin's Lymphomas** are commonly known as Hodgkin's Disease (HD) and is classified into following sub-types:

- Lymphocyte predominant
- Nodular sclerosis.
- Mixed cellularity
- Lymphocyte depletion.

Non-Hodgkin Lymphomas (NHL) have been classified in several ways. Currently most accepted is the International Working Formulation. It is reproduced below:

A. Low Grade
   a. Malignant lymphoma, small lymphocytic
   b. Malignant lymphoma, follicular, predominantly small-cleaved cell
   c. Malignant lymphoma, follicular, mixed small cleaved and large cell

B. Intermediate Grade
   a. Malignant lymphoma, follicular, predominantly large cell
   b. Malignant lymphoma, diffuse, small cleaved cell
   c. Malignant lymphoma, diffuse, mixed small and large cell
   d. Malignant lymphoma, diffuse, large cell

C. High Grade
   a. Malignant lymphoma, large cell, immunoblastic
   b. Malignant lymphoma, lymphoblastic
   c. Malignant lymphoma, small non-cleaved cell

D. Miscellaneous
   a. Composite malignant lymphoma
   b. Mycosis fungoides
   c. Extramedullary plasmacytoma
   d. Histiocytic lymphoma
   e. Unclassified
   f. Others

The most recent classification is the Revised European American Lymphoma Group (REAL) classification. A WHO modification of this classification is under review. This classification includes Hodgkin's disease and other lymphoid malignancies as well. It is reproduced below:

A. B-Cell Neoplasms
   I. Precursor B- cell Neoplasms
   Precursor B-lymphoblastic lymphoma/ leukaemia
   II. Peripheral B-Cell Neoplasms
   1. B-cell chronic lymphocytic leukaemia/ prolymphocytic leukaemia/ small cell lymphocytic lymphoma
   2. Lymphoplasmacytoid lymphoma/ immunocytoma
   3. Mantle cell lymphoma
   4. Follicular centre cell lymphoma
   5. Marginal zone lymphoma
   6. Splenic marginal zone lymphoma
   7. Hairy cell leukaemia
   8. Plasmacytoma/plasma cell myeloma
   9. Diffuse large B-cell lymphoma. Sub-type Primary mediastinal B-cell Lymphoma
   10. High grade B-cell lymphoma, Burkitt like

B. T-Cell Neoplasms
   I. Precursor T-Cell neoplasms
   Precursor T-lymphoblastic leukaemia/ lymphoma
   II. Peripheral T-Cell and NK-Cell Neoplasms
   1. T-Cell chronic lymphocytic leukaemia/ prolymphocytic leukaemia
   2. Large granular lymphocytic leukaemia, T-Cell type & NK-Cell type
   3. Mycosis fungoides/ Sezary syndrome
   4. Peripheral T-Cell lymphoma
   5. Angio-immunoblastic T-Cell

lymphoma
6. Angiocentric lymphoma
7. Intestinal T-Cell lymphoma
8. Adult T-Cell lymphoma
9. Anaplastic large cell lymphoma. Ki-1 lymphoma
10. Anaplastic large cell lymphoma, Hodgkin's like

C. Hodgkin's Disease

1. Lymphocytic predominance
2. Nodular sclerosis
3. Mixed cellularity
4. Lymphocytic depletion
5. Lymphocytic rich classical HD

PLASMA CELL DYSCRASIAS

Plasma Cell Dyscrasias are a group of mature B-Lymphoid cell malignancies, which are now usually classified together with other lymphoid malignancies. These are further classified on the basis of monoclonal protein, which they synthesize. Most important malignancies included in this group are:

- Multiple myeloma
- Waldenstrom Macroglobulinaemia

Multiple myeloma is characterised by accumulation of abnormal plasma cells in the bone marrow and other tissues commonly causing osteolytic lesions and producing abnormal monoclonal immunoglobulin.

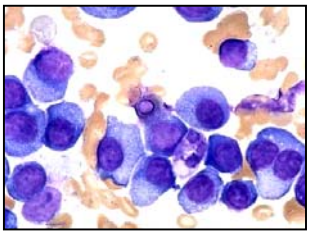
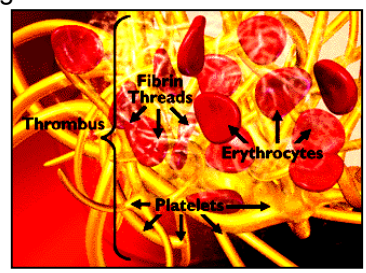


Figure 33.25: Multiple myeloma

Waldenstrom macroglobulinaemia is characterised by the presence of abnormal lymphoplasmacytoid cells in the bone marrow and increased production of abnormal IgM.

HAEMOSTASIS

Haemostasis literally means "stoppage of blood flow". There are three basic components of haemostasis: extravascular, vascular and intravascular. The **extravascular** component is mainly the pressure exerted on the blood vessels because of accumulation of extravasated blood in the tissue space. The efficiency of this component depends upon the bulk of surrounding tissue, the type of tissue and the tone of tissue. The **vascular** component constitutes the blood vessels themselves. The role played by the blood vessels depends upon their size, the amount of smooth muscle in their wall and the integrity of the lining endothelium. On injury the blood vessel undergoes vasoconstriction as a neurogenic response thus decreasing the blood flow. Together with extravascular component it may stop the blood flow altogether. The injury exposes collagen and tissue factor that initiate the participation of **intravascular** components of haemostasis. The key components in intravascular haemostasis are the **platelets**, the coagulation factors, anticoagulants and fibrinolytic factors. Platelets and **coagulation factors** promote formation of thrombus, which occludes the injured site, and result in arrest of bleeding. **Anticoagulant proteins** help in limiting the thrombus formation to the site of injury whereas **fibrinolytic factors** help in dissolution of the thrombus. A fine balance between these keeps the blood in fluid state. A tilt of the balance to one or other side may result in failure of coagulation leading to a bleeding disorder or increased propensity to coagulation leading to Hypercoagulable State or thrombosis.



Exposure of collagen in the wall of blood vessel, following injury, provides surface for adhesion of platelets. The platelets that adhere to this surface undergo **metamorphosis** and a release reaction, which attracts more platelets leading to aggregation of platelets resulting in the formation of a **platelet plug**. Numbers as well as functional integrity of platelets affect this phase in haemostasis. This primary platelet plug is strengthened by the formation of fibrin threads and is converted into a **thrombus**. Fibrin formation is initiated in two ways. First the injury to vessel wall leads to exposure of tissue factor (TF) or **factor III** with which combines a plasma protein, factor VII, and initiates **extrinsic pathway** of coagulation. The exposure of negatively charged elements of the vessel wall (collagen) activates another protein, factor XII, which initiates the **intrinsic pathway** of coagulation. The two pathways converge on a **common pathway**, activating factor X that, in turn complexes with activated factor V. This complex converts prothrombin in the plasma into thrombin, which then polymerise fibrinogen in the plasma to fibrin threads. These threads are then stabalised by the action of activated factor XIII. In this cascade platelets also play a part by providing phospholipid. The details are shown in Figure 33.26. In all, there are 12 proteins and one metal ion (Ca^{++}), which participate in coagulation process. These can be

divided into three groups with similar properties.

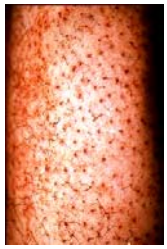
1. **Contact group:** This includes Prekallikrein, High Molecular Weight Kininogen (HMWK), factor XII and factor XI. These are activated on exposure to negatively charged surfaces. These are also involved in fibrinolysis and complement system. The site of their synthesis, apart from factor XI that is synthesised in liver, is not clear. These are all serine proteases.
2. **Prothrombin group:** This group includes factors II, VII, IX and X. These are all serine proteases and are synthesised in liver. These require vitamin K for γ carboxylation of glutamic acid residues to convert these into pro-enzymes.
3. **Fibrinogen group:** This group includes factors I, V, VIII and XIII. Of these I, V and XIII are synthesised in liver.

The activation of coagulation system simultaneously brings into play another set of proteins that have an opposing effect. That is these obstruct the process of coagulation and prevent the extension of clot beyond the required limits. Most important proteins of this system are **Tissue factor pathway inhibitor** (TFPI), **Antithrombin** (AT), **Protein C** and **Protein S**. Another group of proteins, which are collectively termed **fibrinolytic system**, regulates the deposition of fibrin and its removal. The major protein of this system is **plasmin** that is produced by the action of plasminogen activators on a protein named plasminogen, which is synthesised by liver. The most important plasminogen activator is tissue plasminogen activator (t-PA) released from the injured endothelium of the vessel wall.

DISORDERS OF HAEMOSTASIS

Based on the physiology of haemostasis described above, the disorders of haemostasis can be grouped into those arising because of:

1. Vascular defects
2. Platelet defects
3. Defects in coagulation pathway
4. Defects in anticoagulant pathway
5. Defects in fibrinolytic pathway
6. Others



Each of these can be subdivided, based on clinical manifestations, into bleeding disorders and hypercoagulable states or thrombophilia. Each sub-group can be further divided, based on aetiology, into hereditary/congenital or acquired disorders.

1. **Vascular defects**: Hereditary connective tissue disorders like **Ehlers-Danlos syndrome** and **Psuedoxanthoma elasticum** are characterised by weak vessel wall and an abnormal collagen that is unable to initiate the platelet adhesion/coagulation, thus leading to easy bruising and haemorrhagic state. A similar defect is acquired in old age (**senile purpura**) and vitamin C deficiency (**scurvy**). Hereditary alterations in vessel wall structure, e.g., **hereditary haemorrhagic telangiectasia** and **cavernous haemangiomas** lead to bleeding disorder due to weak vessel wall. A similar weakness may also result from acquired diseases like diabetes mellitus and amyloidosis. A bleeding disorder may also result from damage to blood vessels by immune process, as in **Henoch-Schonlein purpura** or in the chronic bacterial **infections**. A thrombotic disorder may result from disease of the vessel wall, e.g., atheroma formation and endothelial injury by toxins or viruses.
2. **Platelet defects**: Platelet defects may be quantitative or qualitative. **Thrombocytopenia** (decreased platelet count) is one of the most common causes of a bleeding diathesis. This may result from decreased production or increased consumption. Most important causes of thrombocytopenia are acquired and not hereditary. Of these the most common is autoimmune or idiopathic thrombocytopenic purpura (ATP or ITP). Most important causes of qualitative platelet defects are hereditary. These include **Bernard Soulier syndrome**, **Glanzmann's thrombasthenia**, **von Willebrand** Disease and Storage Pool defects. A similar disorder can also result from aspirin ingestion.
3. **Defects in Coagulation Pathway**: Although defects in this pathway, e.g., increased levels of coagulation factors, may result in Hypercoagulable State, but more important are the defects, which result in a bleeding disorder. These can be hereditary or acquired. Hereditary bleeding disorders constitute the most important group. These occur because of quantitative or qualitative deficiency of coagulation factors. Although a bleeding disorder may occur because of deficiency of any coagulation disorder but the most common are **Haemophilia A** (Factor VIII deficiency) and **Haemophilia B** (Christmas Disease) because of factor IX deficiency. Most important of acquired bleeding disorders are liver disease and **disseminated intravascular coagulation** (DIC). Liver is the site for synthesis of majority of coagulation factors. Extensive damage to hepatocytes will result in

compromised synthesis of coagulation factors leading to their deficiency. Besides, liver produces bile which is required for absorption of vitamin K, which in turn is needed for synthesis of active forms of factors II, VII, IX and X. Liver disease, particularly obstructive, will therefore also cause qualitative deficiency of these coagulation factors leading to a bleeding disorder. Some quantitative and qualitative disorders of proteins of this pathway also result in Hypercoagulable State. The most important of these is a hereditary qualitative defect of factor V, **Factor V Leiden** and **Prothrombin gene mutation** G→A20210.

4. **Defects in Anticoagulant Pathway**: Quantitative deficiencies of proteins of this pathway result in a hypercoagulable state (thrombophilia). The defects are mostly hereditary in nature. Most important of these are abnormalities of AT, Protein C and Protein S.
5. **Defects in Fibrinolytic Pathway**: These defects most commonly result in thrombotic tendency. These may be hereditary or acquired.
6. **Others**: Some disorders that lead either to a bleeding tendency or a hypercoagulable state involve more than one of above groups as well as other elements. Most important of these are **Von Willebrand Disease** (vWD), DIC and autoimmune diseases like SLE. vWD results from abnormality or deficiency of one part of factor VIII complex, von Willebrand factor (VIII:vWF). This part is independently produced by vascular endothelium and is required for platelet-vessel wall interaction. It results in a bleeding disorder that has the features of a disease both due to platelet defect and coagulation protein defect. This is a hereditary defect. DIC clinically manifests mainly as a bleeding disorder with a component of thrombotic state. It results from initiation of uncontrolled coagulation process, which results in **consumption of platelets and coagulation proteins**. This eventually leads to deficiency of coagulation factors as well as thrombocytopenia leading to bleeding disorder. This is an acquired defect. In the course of some autoimmune diseases inhibitors of coagulation or antithrombotic factors are produced and result in either a bleeding or a hypercoagulable state. **Lupus anticoagulant** results in a prothrombotic state whereas factor VIII inhibitor results in haemophilia like disorder.

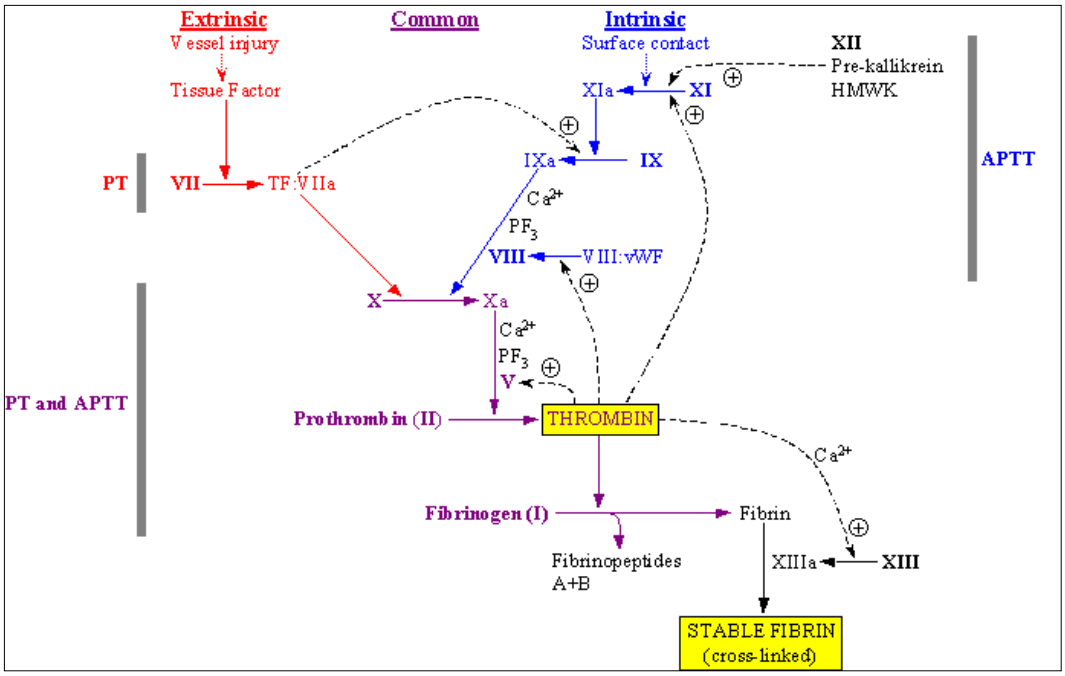


Figure 33.26: The coagulation cascade and laboratory coagulation tests. The pathway *in vivo* begins with activation of factor IX by factor VIIa. The factor XII and pre-kallikrein reactions are probably only relevant *in vitro*. Factor IX is activated by thrombin *in vivo*. Extrinsic pathway, factor VII; Intrinsic pathway, factors XI, IX, VIII; Common pathway, factors X, V, II (prothrombin), I (fibrinogen); -a, denotes activated form of factor; HMWK, high molecular weight kininogen; TF, tissue factor; Ca^{2+}, calcium ions; PF_3, platelet factor 3; vWF, von Willebrand factor. *Coagulation tests*: APTT, activated partial thromboplastin time; PT, prothrombin time

34. BASIC METHODS IN HAEMATOLOGY

ESTIMATION OF HAEMOGLOBIN (Hb) CONCENTRATION

Whole blood haemoglobin concentration can be estimated by a number of methods. Most commonly used methods are:

- Cyanmethaemoglobin method
- Alkaline haematin method
- Acid haematin method

Each of these methods has its advantages and disadvantages. Most commonly used method is cyanmethaemoglobin method. Major advantage of this method is the availability of a stable and reliable standard preparation. This method, however, does not measure sulphhaemoglobin (SHb). Acid haematin method has the advantage of being useful without a colorimeter (Sahli's haemoglobinometer) but is the least accurate of all. Alkaline haematin method has the advantage that it can measure carboxy-haemoglobin, methaemoglobin and sulph-haemoglobin but it does not measure foetal haemoglobins (HbF and Hb Barts').

CYANMETHAEMOGLOBIN METHOD

The principle of this method is that blood sample is diluted in a solution containing potassium cyanide and potassium ferricyanide (Drabkin's solution). It converts haemoglobin (Hb) and methaemoglobin (Hi) to cyanmethaemoglobin (HiCN), which is a stable compound. The absorbance of the solution is measured in a photoelectric colorimeter with a yellow green filter or in a spectrophotometer at a wavelength of 540 nm and is compared with a standard solution of HiCN.

Requirements

1. **Diluent (Drabkin's solution)**

Potassium ferricyanide	200 mg
Potassium cyanide	50 mg
Potassium dihydrogen phosphate	140 mg
Nonidet P40 (Sigma)	1 ml
Distilled water	up to 1000 ml

The *p*H should be between 7.0-7.4 and the solution should be clear and pale yellow in colour. It should give zero absorbance against water at 540 nm. The reagent is stored at room temperature in a brown borosillicate glass bottle. If Nonidet is not available then reaction time is to be increased, as haemolysis may be slow. Reagent can be obtained in prepared concentrate form. If stored properly, the reagent is fit for use for several months. The reagent is discarded if it becomes turbid or the absorbance changes.

2. **Cyanmethaemoglobin reference solution**: The cyanmethaemoglobin reference preparation is used for direct comparison with blood, which is also converted to HiCN. Solutions of different concentrations are commercially available and if unopened are stable for years. But once opened, it is only stable for few hours. It is therefore recommended that a calibration curve should be prepared with the help of these solutions and future readings should be taken from it. But it is necessary that with each batch of tests or at least few times a day the calibration is checked by a fresh cyanmethaemoglobin reference solution or an internal reference prepared against it. The manufacturer's inset with the pack of standards gives the Hb g/L equivalent of HiCN concentration of the standard.

Procedure

Venous blood collected in EDTA or free flowing capillary blood can be used. Measurement can be carried out on blood that has been stored at 4°C for several days, provided it is free from infection and contamination. 20 µl of blood is added to 4 ml of diluent and well mixed by inverting the tube several times. It is allowed to stand at room temperature for 3-5 min so that all Hb is converted to HiCN. The absorbance is then measured in the spectrophotometer at 540 nm**.** Hb level can be directly read from previously prepared calibration curve or chart. Alternatively, absorbance of known standard is also read in the spectrophotometer with each batch of tests and Hb is calculated by the formula:

$$\text{Hb (g/L)} = \frac{\text{Abs. of test} \times \text{Conc. of Std. (g/L)}}{\text{Abs. of Std}}$$

Preparation of calibration curve/chart

Commercially available standard solution of HiCN is diluted in Drabkin's solution so as to give concentrations equivalent to Hb concentrations of 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0, 16.0, 18.0 and 20.0 g/dl. Pre-diluted standards are also commercially available.

Absorbance is read in a spectrophotometer at 540 nm. These readings are converted into Hb conc. in g/dl with the help of conversion table provided by the manufacturer of the standard. Absorbance is plotted against Hb concentration on a linear graph paper, absorbance being on vertical axis and Hb conc. on horizontal axis. All points must join in a straight line. A ready reference chart can be prepared from this curve (see also PREPARATION OF CALIBRATION CURVE on page 47).

Precautions

- Performance of equipment and calibration curve should be quality controlled by testing simultaneously a commercial or in-house reference preparation with each batch of tests and maintaining quality control chart. For details see the chapter on quality control.
- If Nonidet has not been added to the diluent, then 10-15 min should be given for reaction to complete and reading should be taken immediately.
- Abnormal plasma proteins and high white cell count may result in turbid reaction mixture. This should be centrifuged and clear supernatant should be used for taking the reading.

SAHLI'S ACID HAEMATIN METHOD[1]

The method is based on the principle that haemoglobin is converted into acid haematin by addition of 0.I N Hydrochloric acid. The resultant solution is then compared with a reference solution in a colorimeter or coloured strip (in Sahli's haemoglobinometer, see page 17). Details of procedure, if a photoelectric calorimeter is used, are the same as for cyanomethaemoglobin method. Details of procedure, when Sahli's haemoglobinometer is used are given below:

Requirements

- Sahli's haemoglobinometer
- Sahli's pipette
- 0.1N HCl
- Dropping pipette

Procedure

1. Fill the tube of Sahli's haemoglobinometer up to mark with 0.1N hydrochloric acid.
2. Venous or capillary blood may be used. The Sahli's pipette is filled up to the 20 mark by gentle suction. Wipe outer side of pipette clean. There should be no air bubbles in blood column.
3. Blow the blood into the graduated tube of the Sahli's haemoglobinometer and suck the solution in and out of pipette 2-3 times.
4. Allow to stand for 5 min, so that haemoglobin gets converted into acid haematin.
5. Compare the colour of the solution in the graduated tube with that of the reference strips on either side of the haemoglobinometer.
6. If the colour of the graduated tube is darker, add drop by drop either 0.1N HCl or distilled water by the dropping pipette and mix with glass rod, until the colour matches with the reference strips.
7. Note the reading on the graduated tube. This is the haemoglobin level in g/dl. Some tubes also give level in percentage. To convert percentage into g/dl multiply the percent figure by 0.146.

Reference range:

Adult male: 13.0-17.0 g/dl
Adult female: 12.0-15.0 g/dl

DETERMINATION OF TOTAL RED BLOOD CELL COUNT (TRBC)

The number of erythrocytes present in one litre of blood is the total red blood cell count. The recommended reference method for RBC counting is by using an automated haematology analyser. RBC counting by visual method is cumbersome and gives inaccurate results. Therefore the absolute values calculated from this count are also inaccurate and of little clinical value. Visual method is described here to highlight the visual counting procedures and for those who still do not have access to an automated haematology analyser. For automated method, see Particle (Cell) Counting on page 56.

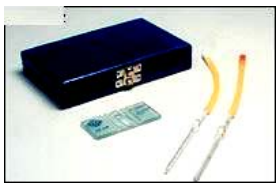
Requirements

1. RBC pipette with a bulb containing red bead as in haemo-cytometer or a Sahli's pipette graduated to 20 µl, or any automatic pipette capable of measuring 20 µl volumes and a test tube.

2. Improved Neubauer chamber with cover slip. It is a thick glass slide with H shaped moats in it. Area between 2 limbs of H is 0.1 mm lower than area on sides. When a cover slip is fixed across these limbs a depth of

[1] Estimation of haemoglobin by Sahli's haemoglobinometer is an inaccurate method and should only be used when photoelectric colorimeter is not available.

0.1 mm is provided in the centre. Above and below the horizontal moat is the ruled area. Moat prevents mixing of two samples charged on either side (Figure 34.1).



3. Red cell diluting fluid. Prepared by dissolving 3.2 g of sodium citrate and 1.0 ml commercial formaldehyde solution in 100 ml distilled water.

4. Microscope

Procedure

1. Draw well mixed blood in RBC pipette up to mark 0.5. Care should be taken not to have air bubbles in blood column (Blood can also be collected from a finger prick as well, Wipe the outer side of the pipette clean.
2. Draw RBC diluting fluid up to mark 201 (1/200 dilution).
3. Gently rotate the pipette between thumb and forefinger to mix well.
4. Alternatively prepare 1/200 dilution of blood in diluent in a test tube by adding 20 µl of blood to 4 ml diluent.
5. Place the cover slip firmly on the Neubauer chamber. The sign of correct placing is that diffraction rings are seen on either side.
6. Discard the first 4-5 drops from the RBC pipette before charging the chamber. Blood diluted in test tube can be used as such after mixing.
7. Charge one side of the chamber by introducing a small drop of diluted blood at the edge of the cover slip. The sample will move under the cover slip by capillary action.
8. Wait for two min to allow the cells to settle.
9. Count the cells using X40 objective in the central large doubly ruled square of the Neubauer chamber. Select 5 small squares, four on corners and one in the centre for counting. At least 500 cells should be counted. If cells are not sufficient in 5 small squares then include more squares for counting and modify the calculations accordingly.

Calculation

Total ruled area of Neubauer chamber is 3x3 mm, divided into 9 large squares, each with an area of 1 mm^2. Central Square is further divided into 25 squares, each of an area of 0.04 mm^2.

Depth of the chamber	=0.1 mm
Thus, volume of a small square	=0.04 x 0.1
	=0.004 mm^3
	=0.004 µl
Volume of 5 small squares	=0.004 X 5
	=0.02 µl
Cells in 5 small squares	=N
Dilution used	=1 in 200

$$\text{Then TRBC per litre} = \frac{N \times 200 \times 10^6}{0.02}$$

Reference range

Adult male = 4.5-5.5x10^{12}/L
Adult female = 3.8-4.8x10^{12}/L

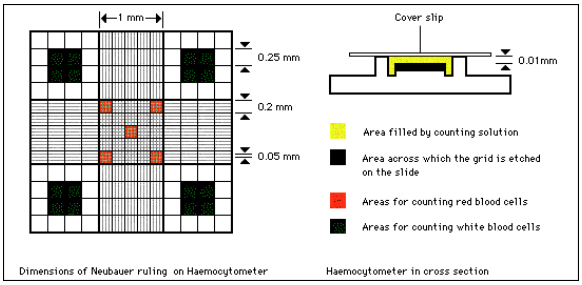


Figure 34.1: Haemocytometer and Neubauer chamber markings

DETERMINATION OF PACKED CELL VOLUME (PCV) OR HAEMATOCRIT (Hct)

When anticoagulated blood is centrifuged, RBCs are packed at the bottom of the tube into a compact mass. These packed RBCs can be expressed as volume of RBC per unit volume of centrifuged blood (L/L) termed packed cell volume (PCV). The packed cells can also be expressed as percentage of total volume of blood centrifuged (%) termed as Haematocrit (Hct). These parameters can be determined by automated equipment or manually using a centrifuge. Manually the packed cell volume can be estimated either by macro method or micro method.

MACRO METHOD (WINTROBE'S METHOD)

Macro method (Wintrobe's method) is no longer in routine use and has been replaced with micro method. However it is being retained for the benefit of those who still do not possess a microhaematocrit centrifuge.

Requirements

- Wintrobe tubes
- Centrifuge with internal radius of 15 cm
- Pasteur pipette with a long capillary end for filling the Wintrobe tube.

Procedure

- Fill the Wintrobe tube up to mark 100 with EDTA anticoagulated well-mixed venous blood. Care should be taken not to introduce air bubbles.
- Centrifuge it at 2000-2300 g (3500 rpm in a centrifuge with internal radius of 15 cm) for 30 min.
- Gently take the tube out of the centrifuge and note the level of upper margin of red cell layer. Buffy coat is not to be included.
- If PCV is above 0.5 L/L, centrifuge the tube for another 30 min and take the reading.

Advantages

- ESR can be read in the same tube first and then centrifuged. (Wintrobe method for determination of ESR is no longer in clinical use and has been replaced with internationally recommended Westergren method).
- No special centrifuge or reading device is required.

Disadvantages

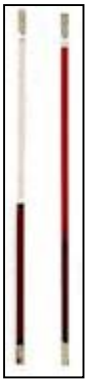
- Larger volume of blood is required.
- Filling and washing of Wintrobe tubes is cumbersome.
- Centrifugation time is long.
- Method is not as accurate as micro method.

MICRO METHOD

International Council on Standardisation in Haematology (ICSH) recommends the micro method for the determination of PCV/Hct.

Requirements

- Heparinised (for capillary blood) or plain (for anticoagulated venous blood) capillary tubes 75 mm in length and 1 mm bore
- Micro haematocrit centrifuge to provide a centrifugal force of 12000g (Figure 34.2)
- Micro haematocrit reader
- Plasticin



Procedure

1. Fill suitable capillary tube with blood. Preferably each sample should be run in duplicate as breakage and leakage of capillary tubes is not uncommon.
2. Seal one end of the tubes with plasticin and place these in the microhaematocrit centrifuge.

3. Centrifuge for 3-5 min.
4. Take out the tube and place in the holder of microhaematocrit reader in such a way that the base of the packed red cells is in line with the base line (0 scale) of the reader and the upper layer of plasma is in line with the slanting line (100 scale).
5. Now adjust the sliding line so that it cuts between the red cell layer and the buffy coat. Note the reading. This is the packed cell volume.

Advantages

- Lesser amount of blood is required. Even the capillary blood can be used making the method convenient for screening for anaemia.
- Less time is consumed.
- Several samples can be run simultaneously.
- Plasma trapping is less.
- It is so correct that it can be used for calibrating automated blood counters.

Figure 34.2: Microhaematocrit centrifuge

Disadvantage

Special equipment is required.

Sources of error

- Sampling error
- Incorrect anticoagulant concentration
- Variation in the bore of the tube
- Incorrect mixing
- Storage for 6-8 hours
- Incorrect filling of tube
- Incorrect centrifugation
- Haemolysis
- Incorrect reading
- Clots in blood sample
- Variation in internal diameter of tube

CALCULATION OF RED CELL INDICES (ABSOLUTE VALUES)

Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) are generally referred to as **Red Cell Indices** or **Absolute Values**. A recent addition is the calculation of RDW. These form the basis for the morphological classification of anaemias.

Absolute values are best determined by automated haematology analysers but can be calculated from the following measured parameters:

- Total red cell count (expressed as count $x10^{12}$/L).
- Packed cell volume (expressed as L/L)
- Haemoglobin concentration (expressed as g/L)

MEAN CORPUSCULAR VOLUME (MCV)

This can be calculated using following formula if PCV and the TRBC are known:

$$\text{MCV in femtolitres (fl)} = \frac{\text{PCV (L/L)}}{\text{TRBC } (\times 10^{12}\text{/L})} \times 1000$$

Reference range

Adult (both sexes): 83-101 fl

MEAN CORPUSCULAR HAEMOGLOBIN (MCH)

This can be calculated by using following formula if Hb and TRBC are known:

$$\text{MCH in picograms (pg)} = \frac{\text{Hb (g/L)}}{\text{TRBC } (\times 10^{12}\text{/L})}$$

Reference range

Adults (both sexes): 27.0-32.0 pg

MEAN CORPUSCULAR HAEMOGLOBIN CONCENTRATION (MCHC)

This can be calculated using the following formula if the Hb and PCV are known:

$$\text{MCHC in g/dl} = \frac{\text{Hb (g/L)}}{\text{PCV (L/L)} \times 10}$$

Reference range

Adults (both sexes): 31.5-35.0 g/dl

Note:- MCH is more reliable when obtained from an automated counter, as RBC count and Hb are more accurate. On the other hand, MCHC can be more reliable in a manual system as this is calculated by Hb and Hct and both of these can be measured accurately by manual method.

DETERMINATION OF TOTAL LEUCOCYTE COUNT (TLC)

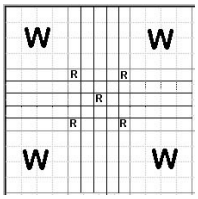
Total Leucocyte Count (TLC) per litre of blood is also best estimated by an automated haematology analyser. However it can also be estimated by visual method. Visual method can also be applied for estimation of cell counts in samples other than whole blood, e.g., CSF (page 95), body fluids, cell cultures or cell concentrates etc.

Requirements

- WBC pipette with a bulb containing white bead, as in haemocytometer or an automatic pipette capable of measuring 50 µl fluid.
- Improved Neubauer chamber with cover slip
- WBC diluting fluid prepared by mixing 4 ml glacial acetic acid and 10 drops of 0.3% aqueous solution of methylene blue and making the volume to 200 ml with distilled water. Methylene blue stains the nuclei of WBC while glacial acetic acid destroys the red blood cells.
- Microscope

Procedure

1. Draw the blood in WBC pipette up to 0.5 mark. Wipe clean the outer side of the pipette.
2. Then draw diluting fluid up to mark 11.
3. Mix gently by rotating pipette between thumb and forefinger.
4. Alternatively draw 0.1 ml well mixed anticoagulated blood in an automatic pipette and add it to a test tube containing 1.9 ml of diluting fluid.
5. Place cover slip on the Neubauer chamber and fix it as described in TRBC procedure.
6. Charge the chamber after discarding 2-3 drops of diluted blood.
7. Let it stand for 5 min so that the cells settle down.
8. Count white blood cells, by using high dry (x40) lens in the 4 large corner squares of the Neubauer chamber (Figure 34.1). Cells on the left and bottom lines are counted whereas cells on the right and top lines are not. At least 100 cells should be counted, even if number of squares to be counted is to be increased.



9. Calculate the mean cell count in a single large square by dividing number of cell counted in 4 large squares by 4.

Calculations

Area of large square	=1 mm^2
Depth of Neubauer chamber	=0.1 mm
Volume of one large square	=0.1 mm^3
	=0.1 µl
Dilution of blood	=1 in 20
Mean number of cells counted	=N

$$\text{TLC/L} = \frac{N \times 20 \times 10^{6}}{0.1}$$

$$= N \times 200 \times 10^{6}$$

$= N \times 0.2 \times 10^9$

Reference range

Adult (both sexes): 4-11x10^9/L

Precautions

1. Pipette should be dry and clean.
2. Dilution should be correct.
3. If liquid flows into the moat, recharge the chamber and count again.
4. Debris of RBC should not be confused with WBC.
5. Cells sticking to debris should be recognised.
6. If nucleated RBC are present in differential leucocyte count then correct the TLC as follows:
   - Count NRBC/100 WBC in DLC
   - Correct TLC by using following formula:

$$\text{Corrected TLC} = \frac{100 \times \text{Observed TLC}}{100 + (\text{NRBC}/100\text{WBC})}$$

DETERMINATION OF PLATELET COUNT

Like other formed elements of blood platelets can also be counted by:

- Electronic particle counter.
- Direct visual method.

Direct visual method is quite reliable and all abnormal platelet counts with electronic counter need to be confirmed by this method. Method recommended by ICSH is described in detail below:

Requirements

1. Diluting fluid 1% ammonium oxalate is recommended. It is prepared by dissolving 1 g dried ammonium oxalate in 100 ml glass-distilled water. Solution is filtered through micropore filter (0.22 µm) and stored in refrigerator.
2. Improved Neubauer chamber with cover slip
3. WBC diluting pipette or 0.1 ml and 1.9 ml automatic pipettes
4. Test tube
5. Moist chamber or a petri dish with moist cotton or tissue paper

Procedure

1. Make 1 in 20 dilution of the whole blood sample. If WBC pipette is used then the dilution is made as in TLC. Otherwise mix 0.1 ml of well-mixed EDTA anticoagulated blood to 1.9 ml of diluent in a suitable test tube to make a 1 in 20 dilution and mix well.
2. Fix a cover slip on a clean Neubauer chamber and charge the chamber as described in DETERMINATION OF TOTAL RED BLOOD CELL COUNT (TRBC) on page 250.
3. Now place the counting chamber in a moist chamber or a petri dish with moist cotton (to avoid drying) for 20 min so that the platelets get settled.
4. Place under the microscope and count by using the high dry (x40 objective) lens of the ordinary light microscope, with the condenser racked down and diaphragm suitably narrowed. Platelets are seen as small, highly refractile discs.
5. Count platelets in the central large square (1 mm in area). The total number of platelets counted should be at least 200, even if more squares are to be included in counting.

Calculations

$$\text{Platelet count/litre} = \frac{\text{No counted} \times \text{dilution} \times 10^9}{\text{Volume counted (µl)}}$$

Thus, if N be the number of platelets counted in a volume of 0.1 µl, then the number of platelets per litre of blood

$$= \frac{N \times 20 \times 10^9}{0.1}$$

$= N \times 0.2 \times 10^9$

Reference range

All ages and sexes 150-400x10^9 /L

Precautions

- Water used for preparation of diluent must be particle free and glass-distilled.
- Glassware used must be scrupulously clean.
- Chamber and cover slip must be clean and scratch free.
- Details of the procedure must be carefully followed.
- Careful filling and counting of cells within the chamber.
- Careful mixing of blood and accurate pipetting and counting of cells.

DETERMINATION OF ABSOLUTE EOSINOPHIL COUNT

Absolute eosinophil count is some times requested either in blood or in other body fluids and secretions. Details of the method for counting are the same as for TLC. The diluent is however different. A suitable diluent is as under:

Acetone	10 ml
Distilled water	90 ml
Eosin	01 g

DETERMINATION OF RETICULOCYTE COUNT

Reticulocytes are immature red cells. These contain thread like structures in the cytoplasm, which consist of ribonucleic acid (RNA). RNA

has the property of reacting with certain dyes such as brilliant cresyl blue or new methylene blue (**supravital stains**) to form a blue or purple precipitate of granules or filaments (Figure 33.2). New methylene blue stains the RNA filaments more deeply and uniformly and should be preferred. The number of reticulocytes in the peripheral blood represents the erythropoietic activity.

Requirements

- Reticulocyte stain: Take 1.0 g of new methylene blue or brilliant cresyl blue and dissolve in 100 ml of citrate saline solution (0.049 g trisodium citrate dissolved in 100 ml of normal saline). Filter the mixture and it is ready for use.
- Pasteur pipette
- 75x10 mm plastic test tube
- Microscope glass slide
- Incubator or water bath at 37°C
- Spreader
- Microscope

Procedure

1. Deliver 2 or 3 drops of stain by means of Pasteur pipette into test tube. Add to it 2-3 drops of the patient's EDTA anticoagulated blood.
2. Incubate the mixture at 37°C in a water bath or incubator for 15-20 min.
3. Re-suspend the cells by gentle mixing. Prepare smears on glass slides and air dry.
4. When films are dry, examine under a microscope using oil immersion lens.
5. Choose an area of the film where the cells are not distorted or overlapping and are properly stained. Count the reticulocytes and the RBC in the area. The field of counting can be narrowed either by using an eye piece provided with an adjustable diaphragm or inserting a piece of paper or card-board in the centre of which a small square with sides about 4 mm is cut, into the eye piece. At least 100 reticulocytes are counted.
6. Calculate the percentage of reticulocytes. If the number of reticulocytes seen is 100 and total red blood cells present are 2500 then reticulocyte count is equal to:

$$\frac{100 \times 100}{2500} = 4\%$$

7. This can be converted into absolute reticulocyte count, if TRBC is known, by the following formula:

$$\text{Reticulocytes } 10^9/\text{L} = \frac{\%\ \text{reticulocytes} \times \text{TRBC } (\times 10^{12})}{100}$$

8. It is important to adjust the reticulocyte count according to the degree of anaemia. This is known as the adjusted reticulocyte count. For this purpose optimum haemoglobin is taken as 15 g/dl or a PCV of 0.45 L/L. Then Corrected Reticulocyte Count %

$$= \frac{\text{Observed count (\%)} \times \text{Patient Hb (g/L)}}{150} \text{ OR}$$

$$= \frac{\text{Observed count (\%)} \times \text{Patient PCV (L/L)}}{0.45}$$

Reference range

Adult (both sexes)	0.2-2%
Infants	2-6%

Precautions

1. Reticulocyte count should be done on fresh blood because if blood is stored the reticulocytes will mature leading to a false low count.
2. At least 1000 red cells should be counted.
3. Reticulocytes should not be confused with HbH inclusions found in HbH disease. HbH inclusions stain paler, are dot like and occur in most of the red cells. If there is doubt, the reticulocyte count should be performed again after incubating the red cells and stain solution for another 2-4 hours. If HbH inclusions are present, the count should not decrease.
4. Heinz bodies appear as small dots present near the cell membrane and should not be confused with reticulocytes.

DETERMINATION OF ERYTHROCYTES SEDIMENTATION RATE (ESR)

If a column of anticoagulated blood is allowed to stand vertically in a tube of narrow bore, the red cells settle down towards the bottom of the tube. The rate at which the red cells settle is known as the erythrocytes sedimentation rate (ESR).

ESR can be performed either by Wintrobe's method or by Westergren's method. The Westergren's method is recommended by ICSH. In this method, properly diluted blood sediments in an open-ended glass tube mounted vertically on a stand. The Westergren's method can be performed on blood that has been collected either directly in liquid tri-sodium citrate anticoagulant or in powder EDTA. Four volumes of venous blood are anticoagulated with 1 volume of 3.2 percent trisodium citrate. If EDTA is used as an anticoagulant, then add 1 volume of 3.2% trisodium citrate to 4 volumes of blood before performing the test.

Requirements

1. Westergren tube. It is an open-ended tube, 30 cm in length and has a diameter of 2.55

mm. It is marked from the bottom in mm up to 20 cm length. The bore must be uniform and smooth[1].
2. Westergren stand
3. Rubber teat or a mechanical device for filling the tube.

Procedure

1. Take a Westergren tube and fill it with diluted blood to zero mark with suction applied by a teat or mechanical device.
2. Place a fingertip over the upper end of the Westergren tube to hold the column of blood in the tube.
3. Fix the tube in Westergren stand (Figure 34.3 and allow it to stand there for exact one hour.
4. At the end of one hour read the height of clear plasma to the nearest one mm.

Figure 34.3: Westergren ESR equipment

Precautions

- Westergren tubes must be scrupulously clean and dry. After use these should be thoroughly washed with tap water, then rinsed with acetone and allowed to dry.
- The surface of the table, on which the stand is placed, must be level and vibration free.
- The test should be protected from draught and direct sunlight.
- The test should be carried out at room temperature (18-25°C). Sedimentation is accelerated at high temperature.

Reference range

Males 17-50 years: up to 10 mm in one hour
Female 17-50 years: up to 12 mm in one hour
Newborn ESR is usually low

PREPARATION AND STAINING OF BLOOD FILMS

Examination of a properly prepared and stained blood film constitutes the most important investigation in Haematology. It is performed for:

- Differential leucocyte count (DLC)
- General assessment and verification of various cell counts.
- Study of RBC morphology for classifying various anaemias.
- Study of WBC morphology for diagnosing leukaemias and other WBC disorders.
- Study of platelet morphology for diagnosing some platelet disorders.
- Study of parasites found in plasma or WBC or RBC (haemoparasites).
- Study of other defects like rouleaux formation, agglutination, fragmentation, red blood cell inclusions, WBC inclusions, platelet clumps and satellitism etc.

[1] Disposable Westergren sets consisting of citrate anticoagulant bottle, disposable tubes and graduated stands, with or without reading devices, are commercially available.

PREPARATION OF BLOOD FILM

Blood films can be made on cover slips (Cover slip method) or on glass slides (Wedge technique). Although **cover slip method** provides superior WBC distribution but is not preferred because of following disadvantages:

- Difficult to prepare because of fragility and small size of cover slips.
- Cover slips are difficult to handle, clean and label.
- Platelets are un-evenly distributed between two cover slips.
- There are no specific areas to be examined.

Blood films prepared on **glass slides** using **wedge techniques** are preferred because:

- These are easy to prepare.
- Pre-cleaned slides are available.
- Handling and labelling is easy.
- It is easy to find abnormal cells, as these tend to collect at the tail and on edges of the film.

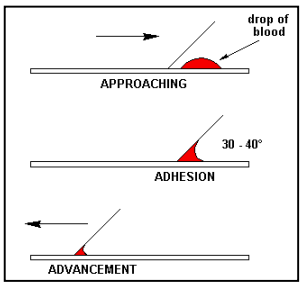
It has some disadvantages as well e.g., greater trauma to cells and uneven distribution of white cells, which tend to collect at the tail.

Requirements

- Pre-cleaned (grease, dust and lint free) glass slides for microscopy.
- Spreader: A spreader is also a piece of glass (cover slip or glass slide). It should be narrower than the glass slide. Its edge should be thin, smooth and polished. The tough cover slip of a Neubauer chamber can serve as an excellent spreader.

Procedure

- Place a small drop of blood in the centre line of the slide, one cm from one end.
- Immediately place a spreader in front of the blood drop at an angle of 45°. Move it back so that it touches the drop of blood. Blood will spread along the margin in contact with slide of the



spreader by capillary action
- Push the spreader forward along the length of the slide by rapid but smooth and straight movement.
- Allow the film to dry in air.

Characters of a good blood film
- It covers at least half the length of glass slide.
- It is narrower than the slide.
- It is spread homogeneously with gradual transition from thick to thin areas clearly identifiable into a head (thick part near the blood drop), body (middle part) and a tail (thin terminal part) (Figure 34.4).
- It has no bubbles, streaks, troughs or holes.
- It terminates into a smooth, straight or slightly curved end.
- It is thin enough to yield at least 10 low power fields where RBCs do not overlap.

Common defects and their causes
1. Thick film results if blood drop is too large, spreading is done too quickly or the angle of the spreader is too high.
2. Thin film results if blood drop is too small, spreading has been too slow or angle of the spreader was too low.
3. Gritty tail results if spreading has been too slow, there was a delay in spreading, only a part of blood drop was utilised or spreader was not appropriate. In addition some anticoagulants other than EDTA and high TLC also give rise to gritty tail.

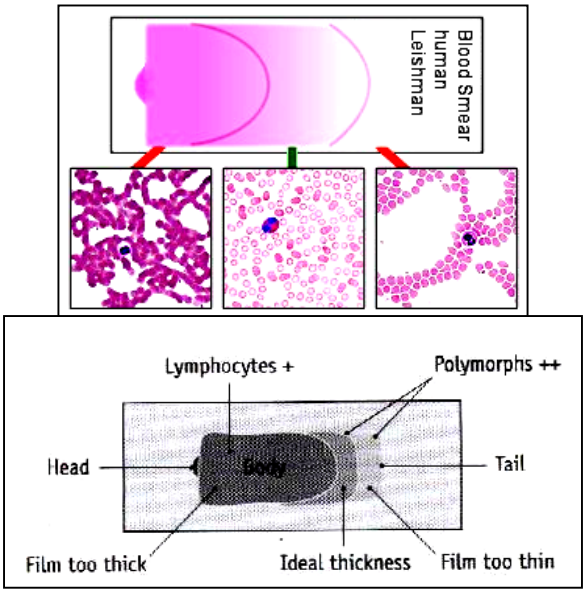


Figure 34.4: Parts of blood film

STAINING OF BLOOD FILMS

Most commonly used stains for staining of blood films are **Romanowsky stains**. These stains are composed of azure B and eosin Y. Azure B combines with anionic components of the cell e.g., DNA and stain these blue, whereas eosin Y combines with cationic components, various proteins and stains them red. Then there occurs a stain-stain interaction. This composition and mode of action allows Romanowsky stains to make clear the subtle differences in shades of staining and allows for differential staining of granules. The *p*H of the staining mixture is extremely important for the differential staining. Alkaline *p*H accentuates the basic dye staining. Therefore, an optimum *p*H is to be sought. A *p*H of 6.8 is recommended for optimal staining of all components. Four most commonly used Romanowsky stains are:
- Jenner's stain
- Wright's stain
- Leishman stain
- Giemsa stain

Leishman stain and May-Grunwald-Giemsa stain are the most frequently used. Preparation and method of use of these is described below.

PREPARATION OF LEISHMAN STAIN

Requirements
- Leishman stain powder of high (at least 80%) purity, 0.2 g
- Methanol (acetone free), 100 ml
- Conical flask
- Funnel and filter paper
- Mortar and pestle

Preparation
- Weigh 0.2 g of powder stain and transfer it to a mortar.
- Grind with about 25 ml of methanol and allow it to settle. Transfer supernatant through filter paper to the flask.
- Add another 25 ml of methanol to mortar containing residual stain. Repeat grinding, allow to settle and transfer the supernatant to the flask.
- Repeat procedure until whole methanol has been used and most of the stain has been dissolved.
- Place the flask in a water bath at 50°C for 15 min.
- Filter into a clean brown borosilicate glass bottle for ripening.
- Leave to mature for at least 2-3 days in the dark at room temperature.

A good practice is to make 2-3 bottles at a time initially. When one bottle is finished, it should be replaced with freshly prepared stain and left to mature. In the mean time other bottle of stain is used. Required volume of stain for daily use should be filtered into a smaller dropping bottle every morning.

PREPARATION OF BUFFER (SORENSEN'S 66 mmol/L)

Preparation

1. **Solution A**: Dissolve carefully weighed potassium dihydrogen phosphate in one litre of distilled water in a conical flask, transfer to a clean glass bottle and store in refrigerator.
2. **Solution B**: Dissolve and store disodium hydrogen phosphate in one litre of distilled water.
3. To prepare buffer of *p*H 6.8, mix 50.8 ml of solution A with 49.2 ml of solution B (page 417).

PREPARATION OF MAY-GRUNWALD-GIEMSA STAIN

Requirements

- May-Grunwald's stain powder of high (at least 80%) purity 0.3 g
- Giemsa's stain powder of high (at least 80%) purity 0.3 g
- Methanol (acetone free) 200 ml
- Conical flasks.

Preparation (also see page 393)

- In a conical flask transfer weighed May-Grunwald's stain powder. Add to it 100 ml of methanol and dissolve.
- In other conical flask transfer weighed Giemsa's stain powder. Add to it 100 ml methanol and dissolve.
- Warm both flasks in water bath at 50°C for 15 min, shaking at intervals.
- Stains are filtered into clean bottles and stored in dark at room temperature.

STAINING OF BLOOD FILMS WITH LEISHMAN STAIN

Requirements

- Prepared Leishman stain
- Buffered water. Dilute 50 ml of Sorensen's buffer of *p*H 6.8 to one litre with distilled water.
- Staining rack

Procedure

- Prepare the blood film and air dry.
- Keep it on a staining rack and cover completely with stain.
- Leave to stain for 2 min.
- Pour buffered water on to the slide about twice the amount of stain. Mix by blowing gently through a pipette. Leave for 5-7 min.
- Pour off stain mixture. Wash in buffer, cleaning the underside of slide with a cotton swab or tissue paper.
- Place vertically to drain and dry.

STAINING OF BLOOD FILMS WITH MAY-GRUNWALD-GIEMSA STAIN

Requirements

- Prepared May-Grunwald's stain
- Prepared Giemsa's stain
- Methanol (acetone free)
- Buffered water (as in Leishman staining)
- Staining jars

Procedure

- Place air-dried blood film in a jar, containing methanol, for 5-10 min.
- Transfer the film to a jar containing May-Grunwald's stain diluted with equal amount of water. Leave for 15-20 min.
- Now transfer the film to a jar containing Giemsa's stain diluted 1:10 with water. Leave for 10-15 min.
- Wash in 3-4 changes of buffered water (pH 6.8) and allow to stand in a jar containing buffered water for 3-5 min for differentiation to take place.
- Drain and dry in vertical position.

COMMON PROBLEMS IN STAINING AND THEIR CAUSES

1 Too red staining is caused if:
   - Stain is too acid (pH <6.4)
   - Buffer has been used in excess
   - Insufficient time has been allowed for staining
   - Excessive washing has been done
   - Blood film is very thin
   - Water used is contaminated, particularly with chlorine.
   - Stain has been too old (methanol converted to fumeric acid)

2 Too blue staining is caused if:
   - Stain is too alkaline
   - Too little buffer has been added
   - Staining time was too long
   - Washing was inadequate

- Water was alkaline
- Blood film was thick
- Blood film had been stored for a long time
- Blood contained increased quantity of proteins
- Blood contained heparin
- TLC was very high
- Haematocrit was too low
- Drying time of blood film was short

3 Film is washed off during staining if fixation is not complete.

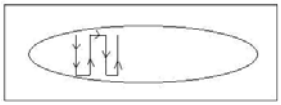
4 Deposit on the slide is seen when stain is allowed to dry on the slide before adding buffer or buffer is not mixed with stain properly

DIFFERENTIAL LEUCOCYTE COUNT (DLC)

Differential leucocyte count (DLC) provides the relative number of each type of leucocyte in blood. It is performed on a well-spread and well-stained blood film. This is of utmost importance, because the even distribution of white cells depends very much upon the meticulous technique used to prepare the blood film and correct identification of cells depends upon quality of staining. If the edge of the spreader is rough, then many leucocytes, especially neutrophils may accumulate at the tail end. If the film is not well prepared or if the film is too thin, neutrophils and monocytes predominate at the margins and the tail, lymphocytes predominate in the middle of the film. A slight difference in distribution is present even in a well prepared film.

Procedure

1. Choose the middle portion of film where cells are evenly spread when seen under the low power of the microscope. Place a drop of cedar wood oil and move oil immersion objective in place.
2. Identify and count each type of cell. Start counting from the thick end of the film and move towards the thin end along a linear strip.
3. When a single strip is completed, then the lens is adjusted to another position vertically upwards or downwards. Counting of the cells is again started, now proceeding in the reverse direction.



4. This procedure is continued until 100 cells have been counted.
5. The counting of cells can be done by:
   - Using a manual or electronic key counter.
   - Writing individual cells and recording the numbers of each cell in division of five.
6. If the count is very high it is better to count 200-500 cells in order to get an accurate idea of the relative number of cells.
7. If there are nucleated red cells present these are not included in the WBC. Instead these are counted separately and reported as number of nucleated red cells/100 WBC.
8. If one basophil appears in 100 cells then another 100 cells should be counted to estimate their correct percentage.
9. DLC is commonly reported as percentage or absolute number calculated from TLC of each type of cell as under
   - Neutrophils
   - Lymphocytes
   - Monocytes
   - Eosinophils
   - Basophils
   - Various maturation stages e.g., blasts, promyelocytes, metamyelocytes and band forms.

   Maturation stages are not normally seen in peripheral blood. Band forms can be seen in peripheral blood and if recorded separately these are normally not more than 6% of the counted cells.

Reference range

Cells	Count x10^9/L	%
Neutrophils	2.0-7.5	40-75%
Lymphocytes	1.5-4.0	20-45%
Monocytes	0.2-0.8	02-10%
Eosinophils	0.04-0.	01-06%
Basophils	<0.01-0.1	<1%

Common problems in cell identification and their causes

1. Too few than expected cells from TLC in the middle portion may result from accumulation of cells at the tail. This results from faulty spreader or improper spreading technique.
2. Difficulty in identifying cells may result from:
   - Poor staining
   - Denaturation of cells
3. Denaturation of cells occur in:
   - Delay in preparation of smears (more than 5 hours for normal cells and one hour for abnormal cells).
   - Improper anticoagulant concentration
   - Blood mixed with IV fluid in the line
   - Patient receiving plasma expanders
   - Severe septicaemia
4. Activation of lymphocytes
5. Vacuolation of monocytes

HESS'S TEST

This test measures the capillary resistance (vascular fragility) as well as any abnormality of platelet number or function. It is a non-specific test and may not always give positive results. It is performed on the patient.

Principle
Impeding venous return raises blood pressure in the capillaries, resulting in small breaches. Normally these are plugged by platelets. But if breaches are more due to increased vascular fragility or if platelets are either less in number or defective in function, then blood extravasates and petechiae appear in greater number.

Requirements
Sphygmomanometer

Procedure
Apply the sphygmomanometer cuff to the arm, and inflate it to 80 mm Hg pressure. Maintain this pressure for 5 min. Inspect the volar surface of the forearm for appearance of petechiae over antecubital fossa. Count the number of petechiae in a 3 cm^2 area. If there are 20 or more petechiae the Hess's test is positive.

Causes of positive Hess's test
- Thrombocytopenia
- Platelet function defect
- Decrease in capillary resistance

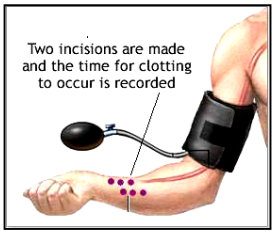
BLEEDING TIME (BT)

Principle
When a standard incision is made on the volar surface of the forearm all mechanisms involved in arrest of bleeding are activated and after some time flow of blood stops. The time taken for the blood to stop flowing, without assistance, from the wound is known as the bleeding time. Bleeding time depends upon the number and function of platelets. If the number of the platelets is reduced below a critical level or these are functionally abnormal, the bleeding time is prolonged. Bleeding time is also prolonged in von Willebrand disease in which, platelet function is disturbed due to absence of vWF.

Requirements
- Sphygmomanometer
- Lancet or template
- Circular filter paper
- Stopwatch

Method
There are two methods by which the bleeding time can be measured:
1. Duke's method. This method is some times used in infants and children.
2. Ivy's method. This is the standard method used.

Duke's method
In this method incisions are made in the ear lobe, pulp of the finger or heel (while it is warm), as these are the sites rich in capillaries.
- Clean the site with a spirit swab.
- Allow the area to dry.
- With the help of a lancet, puncture deeply so that blood flows out freely. Start the stopwatch. At half min intervals blot the drop of the blood at the site of puncture with the help of a filter paper.



- Keep on doing so until blood stops coming out and there is no mark of blood left on the filter paper. At this point stop the stopwatch and note the time. This is the bleeding time.

Ivy's Method
This is the standard method.
- Apply the cuff of the sphygmomanometer to the arm of the patient lying supine on a couch.
- Inflate the cuff to 40 mm Hg. This pressure should be maintained throughout the test.
- Clean the volar surface of the forearm with spirit swabs and choose an area of the skin that does not have any visible veins.
- Make two 4-8 mm long, 1 mm deep, separate punctures along the long axis of the forearm, 5-10 cm apart with standard depth lancet or by a template.
- Let the blood flow out freely and start stopwatch.
- Keep on blotting the oozing blood by gently touching it with edge of circular filter paper at 15 seconds intervals, until the blood stops coming out and no blood spot is left on filter paper.
- Stop the stopwatch and note the time. This is the bleeding time.
- If the bleeding time is more than 15 min and blood is still oozing, stop the test and apply pressure till bleeding is arrested. Write the result as bleeding time more than 15 min.

Precautions
- Check the platelet count before the test. If the count is less than $50x10^9/L$ then test should not be performed.

- There is always a tendency for the wound to close. Therefore, 1 mm deep incision should be made. A superficial incision will result in erroneous results.
- Blood pressure, number and size of incisions must be standardised.
- Area of skin selected for puncture should be clear of visible veins.

Reference range

Dukes' method: 2 - 7 min
Ivy's method (lancet): 2 - 7 min
Ivy's method (template): 2.5 – 9.5 min

Interpretation

1. Prolongation of BT commonly occurs in:
   - Thrombocytopenia
   - von Willebrand disease
   - Platelet function defects
   - Aspirin ingestion
   - Severe deficiency of Factor V or XI
   - Afibrinogenaemia
2. Shortened bleeding time commonly occurs when the technique is faulty.

WHOLE BLOOD CLOTTING TIME

Principle

When blood obtained by a clean venepuncture is put in a glass tube, clotting mechanisms are activated and soon a clot is formed. The time taken by the blood to clot in this way is called whole blood clotting time (CT). Whole blood clotting time is an insensitive and non-specific test. It will be prolonged only in severe haemophilia or Christmas disease, when the factor levels are as low as 1 percent. It is some times used as a bedside procedure to screen for heparin effect and circulating anticoagulants. The Lee and White method is commonly used.

Requirements

- Disposable plastic syringe
- Glass test tubes 75x12 mm (10 mm bore)
- Water bath at 37°C
- Stop watches (3)

Procedure

1. Place three glass test tubes in water bath at 37°C to warm.
2. Clean the venepuncture site with a spirit swab and let it dry.
3. Using a disposable plastic syringe collect 3 ml of blood. As the blood enters the syringe, start all the three stopwatches.
4. Put 01 ml of the blood in each of the three glass tubes already placed in the water bath.
5. Initially tilt the tubes after 4 min and then after every 30 seconds to see whether the blood has clotted or not.
6. When the blood clots in a tube, stop the stopwatch for that tube. Note the time taken by blood to clot for each tube. Take mean of the three readings as result. This is the clotting time.

Precautions

- The venepuncture should be clean and only those samples are to be dealt with, which are obtained after a single prick. This is because by repeated trauma more tissue factor is released and the clotting time may be shortened.
- It is important to start the stopwatch as soon as the blood enters the syringe.
- The tubes should be of specified bore (10 mm) otherwise the result may vary.

Reference range

5-11 min

Interpretation

Clotting time is prolonged in:

- Severe Haemophilia.
- Severe Christmas disease
- Anticoagulant therapy particularly with heparin
- Factor XII deficiency.
- Circulating anticoagulants

PROTHROMBIN TIME (PT)

Principle

Prothrombin time measures the activity of extrinsic and the common pathway of coagulation (factors II, V, VII, X and fibrinogen) under standardised conditions. When tissue thromboplastin and calcium are added to citrated plasma, this pathway is activated and fibrin clot is formed. Time taken by this clot to form is called prothrombin time.

Preparation of thromboplastin

Thromboplastin is freely available commercially and should be preferred as it is pre-standardised. However it can be prepared in the lab from rabbit brain. Rabbit brain preparation, however, is not as sensitive as that of the human brain. But due to danger of AIDS, use of human brain has been abandoned. Method of preparation is as under:

- Sacrifice a rabbit and take out its brain.
- Strip the membranes and the blood vessels from the brain.
- Remove the cerebellum and the brain stem and cut the cerebrum into very small pieces.
- Take about 50 ml of acetone in a mortar and

add to it about 200 g of cerebrum.
- Macerate the brain in acetone.
- Allow to stand. Decant supernatant acetone, add fresh acetone and repeat the procedure.
- Keep on changing acetone until a non-granular powdery material is obtained.
- Collect this powdery material on a clean filter paper and let it dry in a desiccator.
- Once dry, store in small amounts in stoppered tubes at 4°C.
- It is to be freshly suspended in saline (300 mg in 5 ml saline) for use. Warm at 37°C for 15 - 30 min and collect supernatant for use.
- It is important to check the prothrombin time of control plasma by the prepared thromboplastin. If it is more than 14 seconds then more powder is added until the time is adjusted to 14 seconds. If it is less, then dilute with isotonic saline until control plasma gives 14 seconds time.

Requirements
- Patient's platelet poor plasma: Collect 9 volumes of patient blood in one volume of trisodium citrate (31.3 g/L trisodium dihydrate or 38 g/L trisodium pentahydrate) in a plastic tube. Centrifuge at 2000 g for 15 min, preferably at 4°C. Collect platelet poor supernatant plasma into a plastic tube for test.
- Normal control plasma: Prepared by pooling platelet poor plasma obtained from 4-20 normal healthy individuals.
- Thromboplastin: Either commercial or home-prepared thromboplastin can be used. Reagents are commercially available and in some of these thromboplastin and calcium chloride have been combined.
- Calcium chloride (0.025 mol/L) 2.7 g/L
- Glass tubes 75x12 mm
- Automatic micropipettes of 100 µl volume
- Water bath set at 37°C
- Stop watches
- Table lamp

Procedure
- Set the table lamp behind the water bath in such a way that the tubes can be seen against it but the eyes of the technician are protected from direct light.
- Place four plain glass tubes in water bath to warm at 37°C.
- Place a glass tube containing calcium chloride in water bath to warm.
- Deliver 100 µl of test plasma in one of the plain glass tubes.
- Add 100 µl of tissue thromboplastin, mix and wait for one min.
- Then add 100 µl of pre-warmed calcium chloride and start the stopwatch simultaneously. Mix the contents and leave.
- After 6-8 seconds examine the tube against shielded light for clot formation by tilting. Keep on doing so every 1-2 second by briefly taking the tube out of water.
- Stop the stopwatch when a visible clot is formed in the test tube and note the time.
- Repeat the procedure once again on test plasma. Take the mean of the two recorded times.
- Repeat the test on control plasma as for the patient.

Precautions
- Blood should be collected through a clean venepuncture and without much stasis.
- The proportion of anticoagulant and blood should be precise and appropriate.
- The samples should not be allowed to stand at room temperature for long. If a delay is expected, these should be transported on crushed ice.
- Platelet poor plasma should be separated as soon as possible.
- Blood should be collected and processed using disposable plastic syringes, tubes and pipettes.
- The test should always be performed in clean glass tubes.
- Pre-warmed calcium chloride should be discarded at the end of the working session.

Reference Range

10-14 seconds

Result is reported along with controls as below:
- Patient plasma 16 seconds
- Control plasma 14 seconds

Results are also reported as ratio between prothrombin time of patient and test plasma or as INR. These will be discussed later in the manual.

Interpretation
- Prothrombin time is prolonged in deficiency of Factors II, V, VII and X as well as in the presence of heparin. This can occur in following conditions:
- Oral anticoagulant therapy (vitamin K antagonists)
- Obstructive jaundice
- Liver disease
- Haemorrhagic disease of the newborn
- Malabsorption
- Vitamin K deficiency

- Hereditary deficiency of concerned factors
- DIC

PARTIAL THROMBOPLASTIN TIME WITH KAOLIN (PTTK)

Principle
Platelet poor plasma is incubated with kaolin to activate contact phase reactions leading to a clot formation. This measures the overall efficiency of intrinsic pathway of coagulation. It also depends upon the activity of factor II, V and X. Phospholipid is added to provide standardised amount of platelet factor 3 activity and then the mixture is clotted by addition of calcium chloride. Time taken for fibrin clot to appear is noted.

Preparation of Bell and Alton Platelet substitute
- Take 1 g acetone dried brain (prepared for thromboplastin).
- Dissolve in 20 ml acetone and let it stand at room temperature for 2 hours.
- Centrifuge and discard the supernatant.
- Dry the deposit in a desiccator.
- Dissolve in 20 ml chloroform and leave at room temperature for 2-4 hours, mixing time to time.
- Filter and evaporate the filtrate in a desiccator at 37°C.
- Suspend the residue in 10 ml normal saline.
- Determine PTTK of normal pooled plasma with the prepared reagent and adjust concentration to give a PTTK of 35 seconds as was done in thromboplastin preparation.

Requirements
- Test and control plasma is prepared as for prothrombin time.
- Platelet substitute, commercial or home prepared. Some commercial reagents are pre- mixed with Kaolin.
- Kaolin in barbitone buffer *p*H 7.4

Sodium diethylbarbiturate	11.74 g
Hydrochloric acid	430 ml.
Kaolin	2.15 g.

- Calcium chloride as for prothrombin time.
- Automatic micropipettes of 100 and 200 µl volume
- Test tubes 75x12 mm, both plastic and glass
- Stop watches
- Timer
- Table lamp
- Water bath at 37°C

Procedure
- Mix equal volumes of platelet substitute and kaolin suspension and leave in water bath to warm.
- Add calcium chloride into a glass tube placed in water bath to warm.
- Place few 75x2 mm glass tubes in water bath to warm.
- Place 100 µl test plasma in a pre-warmed tube.
- Add to it 200 µl platelet substitute-kaolin mixture. Start the timer and mix at intervals.
- Leave for 10 min in water bath.
- After exact 10 min add 100 µl calcium chloride and start stop watch and mix. Examine for clot formation at intervals as in prothrombin time. Stop the watch as soon as fibrin clot appears and note the time.
- Repeat the procedure on test plasma and take average of the two times.
- Repeat the procedure on normal pooled plasma as for the test plasma.

Precautions
- As for prothrombin time, instructions provided by the manufacturer should be followed.

Reference Range
25-43 seconds, It is better to report against normal control as in PT. Each laboratory needs to determine its own reference range.

Interpretation
- PTTK is prolonged in:
- Deficiency of factors XII, XI, IX, VIII, X, V or II
- Anticoagulant therapy with heparin
- Circulating anticoagulants
- Massive transfusion of stored blood
- Liver disease
- DIC

THROMBIN TIME (TT)

Principle
Thrombin acts directly on fibrinogen and converts it to fibrin. Time taken by clot to form after addition of thrombin is called thrombin time.

Requirements
- Test and control plasma as previously described.
- Thrombin 50 NIH units/ml (commercially obtained).
- Other requirements as for PT and PTTK.

Procedure
- Pre warm few glass tubes in water bath at

37°C.

- Place 200 µl test plasma in a tube.
- Add 100 µl thrombin and start stopwatch.
- Inspect for clot formation and note the time when clot appears.
- Repeat the procedure again and take average of the two times.
- Also observe quality of clot.
- Repeat procedure on control plasma.

Precautions

As described for PT and PTTK

Reference Range

9-11 seconds, Better to report with control

Interpretation

Thrombin time is prolonged in:

- Heparin therapy
- Raised FDPs, Fibrinogen deficiency
- Dysfibrinogenaemia (clot is transparent and bulky)
- Multiple myeloma
- Infancy
- Hypoalbuminaemia

35. BLOOD CELL MORPHOLOGY

Study of morphology of blood cells in a well-spread and well-stained blood film yields invaluable diagnostic information. Therefore the blood film should be examined carefully and systematically. It is preferable that the film should be mounted with a cover glass using a neutral mounting medium. It provides not only good refraction but also preserves the blood film. First it should be examined under low power (x10) objective. This will give an idea of the quality of film and distribution and staining of cells and platelet aggregates. It also gives the idea about rouleaux formation, presence of agglutinates, dimorphic population of cells and presence of some haemoparasites e.g., microfilariae. Then select a suitable area and switch to dry high power (x40) objective. Oil immersion (x100) objective should be reserved for the study of finer details of the cells. There are three types of cells in the blood, RBC, WBC and platelets. Each of these should be studied systematically.

MORPHOLOGY OF RED BLOOD CELLS

Normal red blood cells appear as circular discs of about 6-8.5 µm in diameter, roughly equal to the size of nucleus of a small lymphocyte. They have bright reddish colour (due to haemoglobin) at the periphery, which becomes pale towards the centre because of the bi-concave shape of RBC.

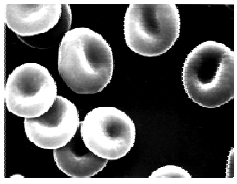


Figure 35.1: Scanning electron microscopy of normal RBCs

The central pale area normally does not exceed one third of the total area of RBC. In a normal blood film RBC lie separately in the central area of the film. RBCs are examined for their distribution, size, shape, colour (Hb content) and inclusions.

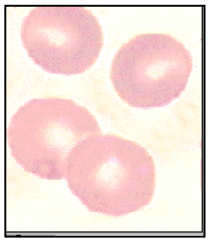


Figure 35.2: Normal RBCs on routine staining and microscopy

Abnormalities in these characters may be artefactual or may arise in disease because of:

1. Changes in plasma proteins and development of antibodies to RBC surface antigens.
2. Abnormal erythropoiesis
3. Inadequate haemoglobin formation
4. Damage to red cells in circulation
5. Increased erythropoiesis

ABNORMALITIES OF DISTRIBUTION

Rouleaux formation: Rouleaux formation (stacking of RBC on top of each other) is seen when fibrinogen concentration of blood is increased e.g., in infections, pregnancy and tissue necrosis. But it is characteristically seen in conditions with abnormal globulin production e.g., in multiple myeloma. The degree of rouleaux formation is directly proportional to ESR.

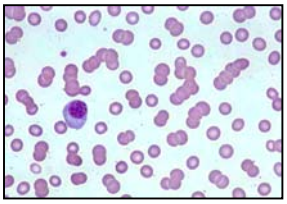


Figure 35.3: Rouleaux formation

Agglutination: Agglutination is defined as random aggregation of RBC. These form clusters of varying number of cells. This results from bridging of cells by antibody molecules, particularly IgM, against antigens on surface of RBC circulating in plasma. These may have been produced endogenously (autoantibodies) as in cold haemagglutinin disease or rarely have been introduced from out side e.g., infusion of large amounts of mis-matched plasma. In incompatible blood transfusion agglutinates seen comprise of cells of donor origin.

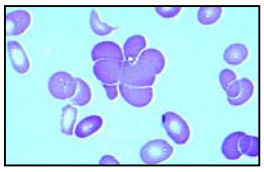


Figure 35.4: Agglutination

ABNORMALITIES OF SIZE

Anisocytosis: If the size of RBC varies, in the same blood film, beyond normal limits, it is termed anisocytosis. It is expressed as + to +++. It is a non-specific feature of several haematological disorders.

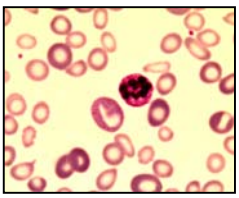


Figure 35.5: Anisocytosis

Microcytosis: When the average size of RBC in a blood film is less than normal it is termed microcytosis. The degree of microcytosis is directly proportional to decrease in MCV. It seldom occurs alone but is usually accompanied

with hypochromia. Microcytosis is commonly seen in iron deficiency anaemia and thalassaemia. Sometimes small cells with no central pale area are seen. These usually have normal MCV. These are termed spherocytes.

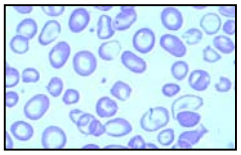


Figure 35.6: Microcytosis

Macrocytosis: When the average size of RBC is more than normal, it is termed macrocytosis. The degree of macrocytosis is directly proportional to increase in MCV . Common causes of macrocytosis are liver disease, megaloblastic anaemia, aplastic anaemia, refractory anaemia, obstructive airway disease, excess of alcohol, treatment with hydroxyurea and hyperglycaemia. In patients whose marrow is responding by increased haematopoiesis and there are lot of polychromatic cells, these appear as macrocytes.

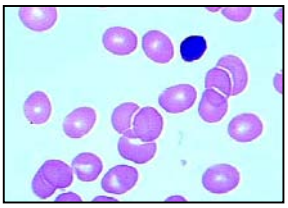


Figure 35.7: Macrocytosis

ABNORMALITIES OF COLOUR

The only true variation in colour is the hypochromia. It results from decreased haemoglobinisation of RBCs, commonly seen in iron deficiency anaemia and thalassaemia. Degree of hypochromia is proportional to MCHC. Leptocytes may appear hypochromic because of flattening. Spherocytes appear hyperchromic because of loss of central pale area and increased thickness of the cell. Macrocytes may also appear hyperchromic because of increased thickness.

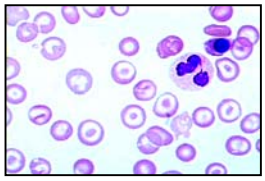


Figure 35.8: Target cells

Target cells: have a central haemoglobinised area, surrounded by a pale ring and then a peripheral haemoglobinised area. These also result from increased membrane surface due to increase in its cholesterol and phospholipid content. These are characteristically seen in thalassaemias, HbC disease, HbD disease, HbE disease, obstructive liver disease, post-splenectomy and iron deficiency anaemia. If an artefact, then these are confined to only a portion of blood film.

Dimorphism: It is the term used when two distinct populations of RBC are seen in the blood film. One population may be normal and the other abnormal, particularly hypochromic microcytic or macrocytic. It is seen in sideroblastic anaemia, when a patient has been transfused or when a patient is receiving haematinics for treatment of deficiency anaemia.

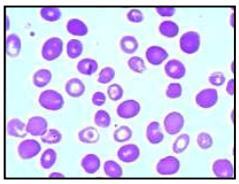


Figure 35.9: Dimorphism

ABNORMALITIES OF SHAPE

Poikilocytosis: When the shapes of RBCs vary more than expected in normal individuals, in the blood film, it is termed poikilocytosis. RBC of abnormal shape is termed a poikilocyte. Poikilocytosis is also a non-specific feature seen in several haematological disorders, abnormal erythropoiesis, megaloblastic anaemia, MDS, iron deficiency anaemia, thalassaemia, and myelofibrosis. However specific types of poikilocytes are diagnostic of specific disorders.

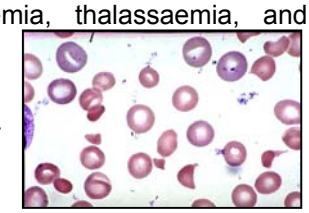


Figure 35.10: Poikilocytosis

Spherocytes: When RBCs are more spheroidal than normal, these are termed spherocyte. These may result from genetic defects of red cell membrane as in hereditary spherocytosis, because of interaction between Ig or complement coated red cells with macrophages as in immune haemolytic anaemias, ABO haemolytic disease of newborn and from action of certain bacterial toxins e.g., *Cl.perfringens*. Spherical forms may be seen when anticoagulated blood is allowed to stand for a long time e.g., banked blood.

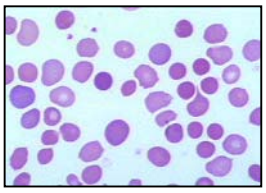


Figure 35.11: Spherocytosis

Elliptocytes and Ovalocytes: About 10% RBC in a normal blood film, particularly at the tail end, appear oval and less commonly elliptical in shape. Their proportion is higher in iron deficiency anaemia, megaloblastic anaemia and myelofibrosis.

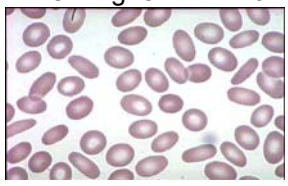


Figure 35.12: Macroovalocytes

In iron deficiency these are usually more elongated (pencil cells), whereas in megaloblastic anaemia these are

macrocytic as well (oval macrocytes).In myelofibrosis ovalocytes are somewhat pointed on narrow side (tear drop cells). If this shape is seen in vast majority of cells and in central area of the film then the condition is termed Elliptocytosis or Ovalocytosis. This results from a hereditary membrane defect.

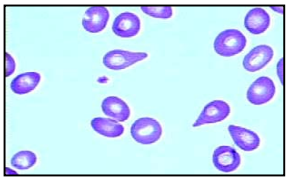


Figure 35.13: Tear drop cells

Stomatocytes: When RBCs have a 'mouth' like slit, these are called stomatocytes. Few stomatocytes are usually seen in normal blood film. Their number is increased in alcoholism, liver disease and Rh null disease. These are numerous in a hereditary membrane defect.

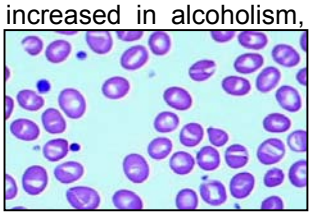


Figure 35.14: Stomatocytes

Schistocytes: These are fragmented red blood cells of various shapes and sizes. Large cells from which portions are fragmented some times appear as helmets and are called helmet cells. Schistocytes are increased in conditions like iron deficiency anaemia, megaloblastic anaemia and thalassaemia but are characteristically increased when RBCs are exposed to mechanical trauma. This occurs when RBCs are passing through meshes of fibrin as in DIC, or through narrowed vessels as in microangiopathy or through prosthesis.

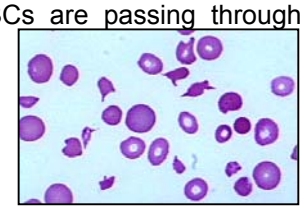


Figure 35.15: Schistocytes

Echinocytes and Burr cells: Echinocytes or crenated cells have evenly distributed blunt spicules of uniform size on their surface. These are formed if anticoagulated blood is allowed to stand for long periods e.g., over night at room temperature or if the film is prepared on a slide that has fatty material on it or if *p*H of the blood is raised.

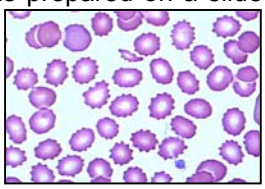


Figure 35.16: Achinocytes

These are also seen in patients who have uraemia or have been on cardiopulmonary bypass. Burr cells are also echinocytes but their spicules are reversible.

Acanthocytes: These are small densely staining RBC with thorn like projections. Generally the projections are fewer, of varying sizes, variable number and more blunted than echinocytes. These may be hereditary or acquired. Hereditary causes include McLeod phenotype and disorders of lipid metabolism. The acquired causes include spur cell anaemia and chronic liver disease.

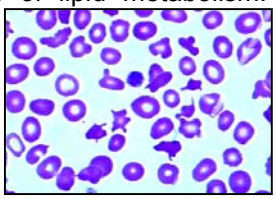


Figure 35.17: Acanthocytes

Pyropoikilocytes:
These are seen in a rare hereditary disorder, pyropoikilocytosis, and comprise microspherocytes and fragments of RBC. Their number greatly increases when blood is heated to 45°C.

Sickle cells: These are thin, elongated, deeply staining red cells with elongated ends. These may be straight, curved or of various other shapes. These are produced by polymerisation of HbS in sickle cell disease.

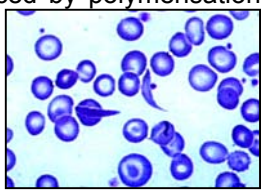


Figure 35.18: Sickle cells

INCLUSIONS IN RBC

Hb crystals: Some abnormal Hb, particularly C and S polymerise to form crystals inside RBC. Polymerisation of HbS gives a distinct shape to RBC, sickle cell. HbS and HbC occurring together polymerise to form straight crystals with parallel sides and one blunt projecting end or multiple crystals projecting from a common centre. HbC crystals are hexagonal with blunt ends. HbH inclusions are shown in Figure 35.19.

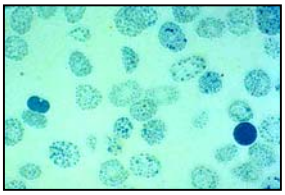


Figure 35.19: HbH inclusions

Howell-Jolly bodies: These are small rounded fragments of the nucleus staining reddish-blue to blue-black resulting from incomplete extrusion of the nucleus. These contain DNA and are <1 μm in diameter. These usually occur singly in RBC but may be multiple. Most common cause is splenectomy or splenic atrophy but these are also seen in alcoholism, sickle cell anaemia, and megaloblastic anaemia.

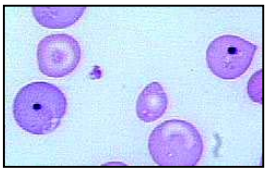


Figure 35.20: Howell-Jolly bodies

Basophilic stippling or punctate basophilia: These are fine to coarse, deep blue to purple, small but multiple inclusions of varying sizes. These represent aggregated ribosomes. These are seen in thalassaemia, megaloblastic

anaemia, liver disease, lead poisoning, unstable Hb, pyrimidine 5-nucleotidase deficiency and infections.

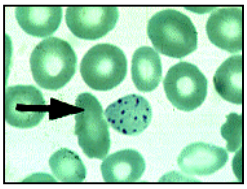


Figure 35.21: Basophilic stippling

Pappenheimer bodies: These are small, dark staining, irregular granules composed of haemosiderin occurring near the periphery of the cells. Their presence is related to iron overload. These stain positively with perl's stain. These are seen in sideroblastic anaemia, dyserythropoietic anaemia and thalassaemia.

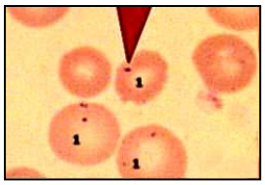


Figure 35.22: Pappenheimer bodies

Cabot rings: This is thin reddish blue, ring like structure occupying varying portion of RBC. It may be twisted to form figure of 8. Its origin is not clear. These are commonly seen in severe anaemia of any type but most commonly in megaloblastic anaemia, lead poisoning and dyserythropoietic anaemias. These may occur alone but are usually associated with punctate basophilia and Howell-Jolly bodies.

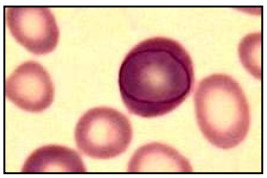


Figure 35.23: Cabot rings

Parasites: These include malarial parasites and Babesia. For details see section on PARASITOLOGY on page 109.

MORPHOLOGY OF WHITE BLOOD CELLS

NEUTROPHILS

Neutrophils are normally the predominant type of WBC in peripheral blood. These are of uniform size, around 13 um in diameter and have a segmented nucleus. These are examined for:

1. Stage of maturation
2. Shape of nucleus and number of lobes in neutrophils
3. Presence and appearance of granules.
4. Cytoplasmic inclusions other than granules.

In normal blood there are hardly any immature forms except up to 8% stab forms (with unsegmented nucleus). Normally neutrophils in peripheral blood have 2-5 lobes but the number of 3-4 lobed cells is more than those with 2 and 5 lobed cells. In females 2-3% neutrophils show an appendage at a terminal nuclear segment, called **drumstick**. It represents the inactive X chromosome. The granules in the cytoplasm of neutrophil are azurophilic, small and evenly dispersed.

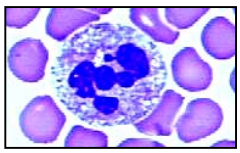


Figure 35.24: Neutrophil showing drumstick

When immature forms appear in the peripheral blood, it is called **left shift**. The simplest left shift is evidenced by increase in percentage of unsegmented neutrophils (stab forms). In more severe case metamyelocytes, myelocytes and even promyelocytes and blasts may appear. Left shift is most commonly seen in severe bacterial infections. Severe left shift, together with increased count is termed **leukemoid reaction**. This should be differentiated from myeloproliferative disorders e.g., chronic granulocytic leukaemia. In leukemoid reaction NAP score (see LEUCOCYTE/NEUTROPHIL ALKALINE PHOSPHATASE (LAP/NAP) on page 277) is characteristically high. Neutrophils with unsegmented nucleus or at the most bilobed nucleus with clear staining of both acidophilic and basophilic contents are called **Pelger cells**. These are characteristically seen in a benign inherited disorder, Pelger-Huet anomaly. Pseudo-Pelger cells are seen in myelodysplastic syndromes, AML and CGL.

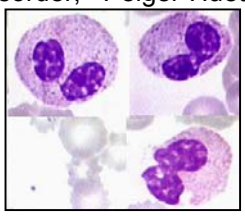


Figure 35.25: Pelger cells

Hypersegmentation: It is defined as increase in proportion of neutrophils with 4 or more lobes of nucleus. Normally 3-lobed nucleus is seen in 40-50% neutrophils, 4 lobed in 15-20% and 5 lobed in <0.5%. It is diagnostic feature of megaloblastic anaemia but may also be seen in uraemia and treatment with cytotoxic drugs (methotrexate and hydroxyurea).

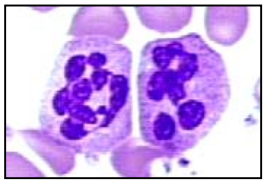


Figure 35.26: Neutrophils showing hypersegmented nuclei

A small number of neutrophils with **pyknotic nucleus** are seen. These are dying cells. Their number increases in infections. Normally cytoplasm contains fine azurophilic granules that are evenly distributed.

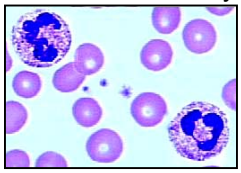
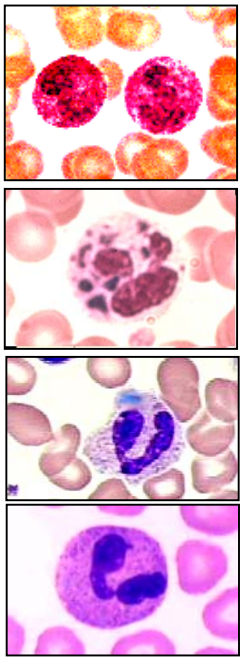


Figure 35.27: Neutrophils chowing toxic granules

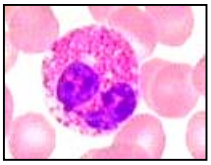
In **toxic granulation** granules are larger,

densely staining and may also be increased in number. **Hypogranular** cells are seen in myelodysplastic syndromes. In rare inherited disorders characteristic granular abnormalities are seen. In **Alder-Reilly anomaly** granules are very large, discrete, stain deep red and may obscure the nucleus. In **Chediak-Higashi syndrome** there are very large (giant) azurophilic granules but are scanty. Sometimes small, round or oval patches of blue colour are seen. These are called **Dohle bodies** These are commonly seen in severe bacterial infections. In a rare inherited disorder **May-Hegglin anomaly** such structures are also seen. In severe infections **vacuoles** of varying sizes may be seen. **Bacteria** may also be seen within these vacuoles.



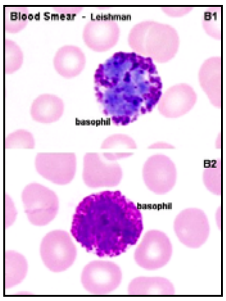
EOSINOPHILS

Eosinophils are slightly larger than neutrophils (12-17 µm in diameter), have a bilobed nucleus and cytoplasm is packed with spherical golden/orange granules. Their number increases in allergic conditions and parasitic infections. Degranulation, hyper-segmentation of nucleus and vacuolation occur as a reactive change. Abnormal granules are seen in CGL, myelodysplasia and AML



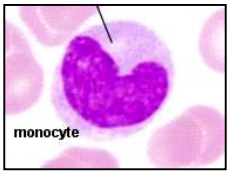
BASOPHILS

These are of the same size as eosinophils. Large, dark blue granules of variable sizes obscure nucleus. The nucleus is usually folded upon itself to appear as compact, irregular and dense. These tend to form vacuoles and degranulate. They are less than 1% in normal blood. Their number is increased in CGL.



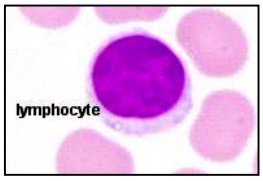
MONOCYTES

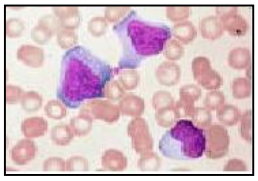
These are the largest or the leucocytes in circulation. They have bluish grey cytoplasm with ground glass appearance and a nucleus, which appears to be folded upon itself. Their number increases in chronic infections and in some types of leukaemias. Reactive change is defined by appearance of vacuoles and spreading ability of the cell. Such cells are called macrophages and may be seen on the margins of blood film in severe bacterial infections.



LYMPHOCYTES

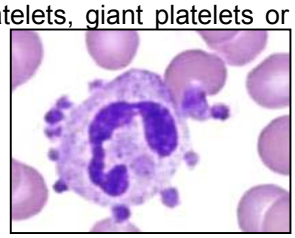
Majority (90%) of lymphocytes in peripheral blood are small with rounded nucleus and a thin rim of cytoplasm occasionally containing few reddish granules. About 10% are large with more abundant cytoplasm and more frequent azurophilic granules in the cytoplasm. Open chromatin and some times with visible nucleoli may appear. These are called **reactive lymphocytes** or **virocytes.** Lymphocytes predominate in the blood films of infants and young children. **Turk cells** are transforming lymphocytes seen in infections. These are slightly larger with a rounded eccentric nucleus, abundant deeply basophilic cytoplasm. In viral infections larger cells with irregular outline and a distinct, round, oval or kidney shaped nucleus.





MORPHOLOGY OF PLATELETS

Platelets are small (1-3 µm) discoid cells, which have no nucleus but a central granulated area. Larger platelets, even ribbons of platelets are seen under conditions of stress (bleeding due to any reason). Platelet count rises in acute inflammation or stress. In myeloproliferative disorders very large platelets, giant platelets or even cytoplasmic fragments of megakaryocytes may be seen. In some individuals, platelets form rosette around neutrophils. This is called **platelet satellitism** and is an antibody-mediated phenomenon. In about 1% EDTA



samples platelets clump together. Characteristic abnormalities may be seen in some rare inherited disorders. In Bernard Soulier syndrome platelets are large (giant) whereas in grey platelet syndrome due to lack of granules these appear grey.

36. BONE MARROW EXAMINATION

In many haematological conditions, particularly leukaemias, examination of the bone marrow may be the only procedure to arrive at a correct diagnosis. It provides the opportunity of examining directly the tissue, which forms blood cells. It is easy to perform and safe, except in severe bleeding disorders like haemophilia, and can be performed as an out-door procedure as many times as required. There are two methods of examining the bone marrow:

- Smears prepared from a needle aspirate.
- Sections prepared from a trephine or open surgical biopsy specimen of the bone marrow.

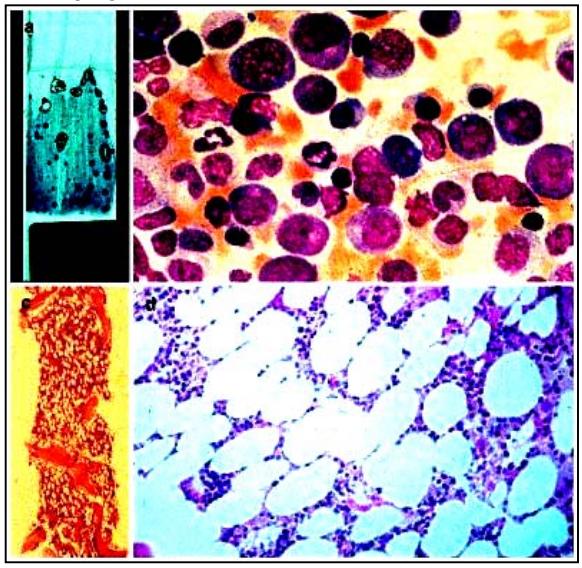


Figure 36.1: Gross and microscopic appearances of bone marrow smear (top) and trephine biopsy (bottom)

Smears show better morphological details of individual cells and are also used for cytochemical staining and immunological studies. However these do not show spatial distribution of normal and abnormal cells and their exact quantity. For this a biopsy section is examined.

BONE MARROW ASPIRATION

INDICATIONS

Needle aspiration of the bone marrow is indicated for diagnosis of primary haematological diseases as well as for diagnosis of certain other illnesses. It is also performed for determining effects of treatment given for some diseases.

A. Diagnostic

- Megaloblastic anaemia
- Aplastic anaemia
- Sideroblastic anaemias
- Iron deficiency anaemia
- Anaemia of chronic disorder
- Acute leukaemia
- Multiple myeloma
- Metastasis in bone marrow
- Storage disorders
- Visceral Leishmaniasis (Kala Azar)
- To obtain haemopoietic cells for cytogenetic studies, molecular genetic studies and immuno-phenotyping.
- Culture for Mycobacteria and other bacteria in cases of PUO.

B. Prognostic

- Staging of chronic leukaemias.
- Staging of lymphomas.
- To determine response of treatment in acute leukaemia and other disorders.

SITES FOR ASPIRATION

Selection of site for bone marrow aspiration depends upon the age of the patient, physique of the patient and expected distribution of disease process. Various sites for bone marrow aspiration include:

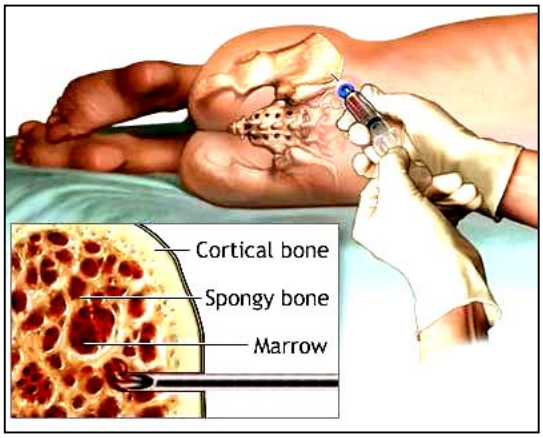


Figure 36.2: Site and structure of iliac crest

1. **Posterior superior iliac spine/crest (PSIS/C)**: This is the most suited site in adults and in children over two years of age. It has the ease of puncturing multiple sites in

one go as well as sampling large volumes of bone marrow. It is safe and as the patient cannot see it, it causes less apprehension to the patient. Bone marrow trephine biopsy can also be performed from this site through the same skin puncture.

2. **Sternum**: The sternum is used in obese but adult patients. It is punctured opposite the second or third intercostal space slightly to one side of the midline. The total thickness of the sternum is about 1.5 cm. Therefore it is necessary that a guard be applied to the needle, so that it should not penetrate more than 0.5 cm of the bone.
3. **Spinous process of vertebrae**: The spinous processes can also be selected for bone marrow aspiration. However, it is necessary that these should be palpable. This is not the site of choice.
4. **Tibia**: In case of children less than 2 years of age anteromedial surface of tibia, slightly below the tibial tuberosity is the site of choice.
5. **Anterior superior iliac spine**: This site may be used in obese patients but is not convenient. First it is more painful, skin being richly supplied with sensory nerves. Second overlying cartilage is thick. Third, it is more acute and contains less marrow, particularly in elderly.
6. **Others**: Occasionally aspiration may be directly performed from a lesion visible in X-ray e.g., lytic lesions in ribs and skull bones.

REQUIREMENTS

1. Salah, Klima or Islam needles are used for aspiration of the bone marrow. First two needles are provided with a guard and are suitable for aspiration from all sites.

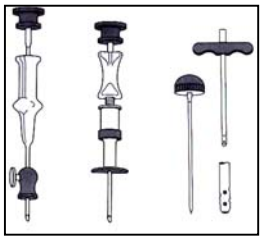


Figure 36.3: Bone marrow aspiration needles

   Islam needle is not provided with a guard but it is longer than others and has holes on the sides that permit collection of better representative sample. It can only be used on PSIS/C.
2. Clean, grease free glass slides, preferably with frosted end for easy labelling.
3. Spreader
4. Large piece of filter paper
5. Disposable syringes of 10 ml
6. Disposable syringes of 20-50 ml with nozzle to fit in the aspiration needle.
7. Antiseptic lotion (0.5% chlorhexidine in ethanol)
8. Local anaesthetic (2% lignocaine)
9. Surgical blade mounted on a handle.
10. Surgical towels
11. Disposable surgical gloves
12. Towel clips
13. Sponge forceps
14. Medicine bowl
15. Sterile surgical gauze

PROCEDURE

1. Prepare the tray or trolley with all the requirements.
2. Place a piece of filter paper and arrange on it 2-3 glass slides in slanting position against a support.
3. Label at least 10 slides with patient identification and arrange for smear preparation.
4. Draw about 5 ml of 2% lignocaine in a disposable syringe and keep aside for later use.
5. Wash hands thoroughly with soap and water and put on surgical gloves.
6. Explain the procedure to patient and reassure him. Patient should be particularly explained about suction pain.
7. Position the patient depending upon the site selected for aspiration.
8. Clean the site with antiseptic solution. A larger area than required is cleaned to prevent infection.
9. Drape the area with surgical towels.
10. Inject lignocaine into the skin, subcutaneous tissues and the periosteum of the bone in about 1-2 cm area. Wait for 3-5 min.
11. Make a small skin-deep nick with blade at the selected site.
12. Introduce the aspiration needle with a gentle boring movement. When the bone marrow is entered there is a feeling of giving away of resistance. Move the needle forward a little more until it is fixed.
13. Remove the stillet and attach a 30-50 ml disposable syringe with the needle.
14. Suck about 0.5 ml of marrow. One of the indications that the marrow has been penetrated satisfactorily is the suction pain.
15. Detach the syringe and replace the stillet.
16. Immediately start making the smears so that the marrow does not clot. Pouring the aspirated marrow on the slanted slides so that free blood is drained while fragments remain stuck on the slide does this. Pick up the fragments with the edge of the spreader and gently smear on the prearranged slides.

17. Put the remaining marrow in an EDTA bottle and mix so that more slides can be prepared if required.
18. Secure haemostasis by firmly pressing the site of puncture for 5-10 min.
19. If required a stitch may be inserted in the nick.
20. Apply dressing.

STAINING OF BONE MARROW ASPIRATE SMEARS

At least two smears should be stained with Romanowsky stain and one with Prussian blue method in all cases. Smears that are well spread and carry enough fragments should be stained. Methods and stains used for Romanowsky staining are the same as described earlier for staining of blood smears. Only difference is that the timings are doubled when Leishman stain is used i.e., slide is covered with pure Leishman stain, which is left in place for 4-5 min instead of two min. Buffer, is then mixed on the slide and it is left for 15-16 min instead of eight min. This is because bone marrow smears are thicker than blood smears and cells being more in number require more time for staining.

PRUSSIAN BLUE STAINING

This is required to stain the iron content of bone marrow. Both intracellular and extracellular iron is stained. The method utilises perl's reaction. In this reaction water insoluble haemosiderin acquires blue colour when exposed to acidic solution of potassium ferrocyanide.

Requirements

1. Fixative: Absolute methanol
2. Acidic potassium ferrocyanide 1%
   a. Solution A: Potassium ferrocyanide 2%: Dissolve 2g potassium ferrocyanide in 100 ml of distilled water.
   b. Solution B: Hydrochloric acid 0.2 mol/L: Add 2 ml of concentrated HCl to 98 ml of distilled water.
   c. Working solution: Mix equal volumes of solution A and B just before use.
3. Counter stain: 0.1% aqueous eosin. 100 mg/100 ml in distilled water.
4. Slide staining jars (Coplin jars)

Procedure

1. Dry the film in air and fix in absolute methanol for 20 min.
2. When dry, place in working solution for 10 min.
3. Wash well in running tap water for about 20 min. Rinse in distilled water.
4. Counterstain with 0.1 percent aqueous eosin for 10-15 seconds.
5. Dry in air and mount.

Result

Iron stains as bright blue granules.

EXAMINATION OF BONE MARROW SMEARS

First examine all the stained slides with naked eye and choose one, which has enough marrow particles (fragments), is spread properly and stained evenly and best. A good smear has a reddish blue colour. In some diseases particles tend to clot during preparation (multiple myeloma, megaloblastic anaemia, autoimmune disorders etc.). In cold haemagglutinin disease RBC agglutinate if the slides and syringe were not warmed before collection and preparation of smears. Then examine the selected slide under low power (x10 objective) of the microscope and note the following:

1. **Cellularity**: This is noted for both fragments and their trails. Normally one third to half of a fragment of bone marrow from an adult contains haemopoietic cells where as rest of it comprises of fat spaces. If the proportion of haemopoietic tissue is less than this then the marrow is termed hypocellular and if it is more then the marrow is termed hypercellular. Some times fragments are only composed of a thick mass of cells. This is some times called **packed marrow**. Similarly the cellularity of the trails is noted. Trail is the part of smear left behind by a fragment during spreading

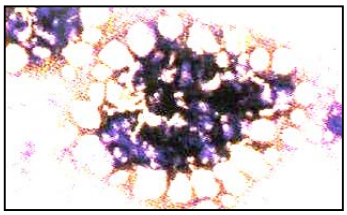


Figure 36.4: Bone marrow fragment (low power)

2. **Megakaryocytes:** Both the number and maturation stages are to be noted. Normally 2-6 megakaryocytes per low power field are present and these contain a nucleus with 6-10 lobes or segments. Megakaryocytes tend to collect towards the tail end and may form masses if the bone marrow has clotted before preparation of smear.

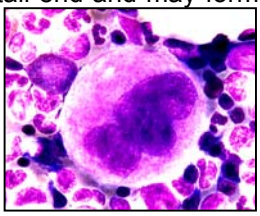


Figure 36.5: Megakaryocyte in bone marrow

3. **Other cells**: At this stage some normal and abnormal large cells may also be seen. Osteoclasts, osteoblasts, macrophages,

mast cells and endothelial cells are normally seen in small numbers but their number may be increased in certain disease states. Osteoclasts should not be confused with megakaryocytes. These are much larger, have granular cytoplasm with indistinct ruffled border and contain several discrete rounded nuclei. Osteoblasts should not be confused with plasma cells. These are oval, have a compact nucleus and cytoplasm stains bluer, has no granules and has a frayed border.

4. **Abnormal cells**: Examine for abnormal cells such as macrophages containing parasites (LD bodies), histiocytes with or without haemophagocytosis, storage cells like **Gaucher** and **Niemann Pick** cells and clumps of malignant cells. Now select a well-stained area and examine with dry high power (x40) objective. Mounted slides are best viewed with this objective. Oil immersion (x100) objective should only be used for differentiating fine details of the cells, if required.

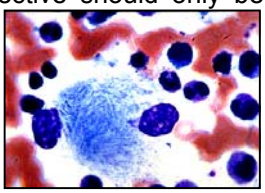


Figure 36.6:

5. **Erythropoiesis**: Note the quantity and quality of erythropoiesis. Whether it is normal, reduced or increased, and whether it is normoblastic or megaloblastic. Also look for dysplastic features e.g., cytoplasmic bridging, nuclear lobulation, multi-nuclearity and fragmentation etc (page 238).
6. **Myelopoiesis**: Note the quantity and quality of myelopoiesis. Particularly look for any change in maturation sequence, granularity of cytoplasm (hypogranular), excess of eosinophilic or basophilic cells and presence of giant myelocytes and metamyelocytes (page 238).
7. **Parasites**: Examine for the presence of parasites, particularly Plasmodium falciparum (page 110), which tend to sequestrate in the bone marrow and Leishmania donovani (page 113).
8. **Myelogram**: Now perform a differential count of all nucleated cells in the bone marrow. This should include all stages of maturation of WBC. However all stages of erythroblasts are counted collectively. At least 500 cells should be counted. It is preferable that differential count should be performed from more than one randomly selected areas. The differential count of bone marrow smear is called Myelogram.
9. **ME Ratio**: Calculate the myeloid erythroid ratio (ME ratio). This is done by dividing the total number of myeloid cells by total number of erythroid cells. Lymphocytes, plasma cells and other non-myeloid and non-erythroid cells are excluded.
10. **Blasts**: If the marrow contains excess of blasts (≥5%) then give their morphological description to differentiate between myeloblasts and lymphoblasts.

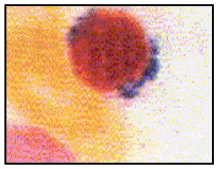


Figure 36.7:

11. **Iron**: Now examine Prussian blue stained smear. First under low power for visible **haemosiderin** in fragments. Slight colour or few granules are normally present. Note whether iron (haemosiderin) is increased or absent. Then examine under oil immersion for quantity and quality of **Siderocytes** and sideroblasts. Siderocytes are mature RBC containing few haemosiderin granules. **Sideroblasts** are erythroblasts containing haemosiderin granules in cytoplasm. Normally in about 40% of polychromatic erythroblasts few small siderotic granules are seen scattered in the cytoplasm. Particularly look for ring sideroblasts at periphery of the fragments. These are erythroblasts in which haemosiderin granules are larger, more numerous and arranged in the form of a ring around the nucleus. These are only seen in disease states e.g., sideroblastic anaemias and MDS.

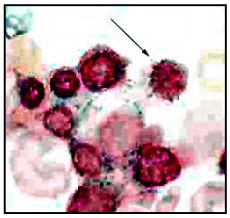


Figure 36.8:

12. **Cytochemistry**: In case of leukaemias further cytochemical stains may be required to differentiate between various FAB types of leukaemias (page 277).

REPORTING OF BONE MARROW SMEARS

A bone marrow report should include patient identification parameters, date of performing the bone marrow aspiration, date of reporting the bone marrow, name of pathologist reporting the bone marrow, site from where the bone marrow was aspirated, consistency of bone (normal, hard or soft) and force required to aspirate (easy, difficult, bloody, dry). After this all observed facts shall be reported sequentially. A typical sequence is description of erythropoiesis, description of myelopoiesis, description of

megakaryocytes, description of lymphocytes and plasma cells, description of abnormal cells including blasts, presence of parasites, description of iron status and finally the ME ratio. It is highly preferable that a myelogram is also included in the report. Finally the report should detail the conclusions drawn, most probable diagnoses and any suggestions regarding further investigations if required.

BONE MARROW TREPHINE BIOPSY

INDICATIONS

- Repeated dry/bloody tap
- Aplastic anaemia
- Myelosclerosis/Marrow fibrosis
- Multiple myeloma
- Hairy cell leukaemia
- Acute megakaryoblastic leukaemia (M7)
- Staging of lymphomas.
- Staging for other tumours (rnetastasis).
- In PUO for granulomas

SITE FOR TREPHINE BIOPSY

Only two sites can be safely used. These are the posterior superior iliac spine and anterior superior iliac spine. The first one is the preferred site.

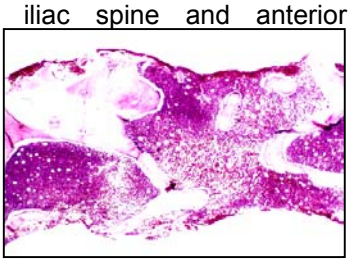


Figure 36.9: Histological section of bone marrow trephine biopsy

REQUIREMENTS

As for bone marrow aspiration, except that a trephine biopsy needle is required in place of aspiration needle and a bottle containing fixative is required. Most commonly used bone marrow trephine biopsy needles are **Jamshidi** (left) and **Islam** (right) (Figure 36.10).

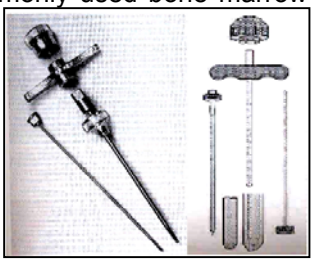


Figure 36.10: Bone marrow trephine biopsy needles

These come in three sizes, a standard adult size, paediatric size and a large size for obese patients. Most commonly used fixative is 10% buffered formal saline as used for other surgical biopsies. Its preparation is described in section on Histotechnology. A preferred fixative is Helly's fluid, which is prepared by dissolving 2.5 g potassium dichromate, 5.0 g mercuric chloride and *5* ml commercial formalin in 100 ml water. Specimen should be left in it for 12-48 hours.

PROCEDURE

1. Prepare the trolley and patient as for bone marrow aspiration. Trephine biopsy may be obtained in the same sitting as for aspiration. Only precaution required is that the site of insertion of trephine biopsy needle in the bone should be slightly away from the site where aspiration needle was inserted. The needle however can be introduced through the same skin incision.
2. After penetrating the periosteum and cortical bone, when the needle gets fixed, the stillet is removed and firm, smooth, regular rotatory movements are performed with pressure to further penetrate to a depth of about 1.5 to 2 cm.
3. To detach the internal portion of the marrow, clockwise and anti-clockwise movements are performed several times without further penetration. After that the needle is withdrawn with the same rotatory movement.
4. Biopsy is dislodged on to a glass slide through the end opposite the penetrating end with the help of stillet to avoid crushing effect.
5. The cylindrical biopsy is gently rubbed against glass slide with the help of another glass slide to make impression smears.
6. It is then put in a specimen bottle containing fixative.

PROCESSING AND STAINING OF BONE MARROW BIOPSY

Fixed bone marrow biopsies are decalcified, dehydrated and impregnated with wax like other histopathology specimen. Then sections are cut and stained as for other tissues. The details are given in section on histotechnology. Two stains are routinely used. These are Haematoxylin-Eosin (H&E) stain and a suitable reticulin stain. Other stains may also be used if required. For demonstrating parasites ideal stain is May-Grunwald-Giemsa stain. However it is difficult to obtain good results with this stain on bone marrow sections. One method, which gives most satisfactory results, is described below.

REQUIREMENTS

- Lugol's iodine: Dissolve 5 g iodine crystals and 10 g potassium iodide in 100 ml distilled water.
- May-Grunwald stain: As described in blood film staining on page 258.
- Giemsa stain: As described in blood film staining.
- Buffered water: As described in blood film

staining.

- Glycerine-ether: Equal volumes glycerine and diethyl ether are mixed.
- Ethanol
- Xylol
- Coplin jars
- Mounting medium
- Cover slip

PROCEDURE

1. Place the sections in Lugol's iodine for two min.
2. Wash thoroughly in tap water.
3. Rinse in buffered water.
4. Dilute May-Grunwald stain with equal volume of buffered water and place the sections in it for one hour.
5. Dilute Giemsa stain with 19 volumes of water (1 in 20) and place the sections in it for two hours.
6. Rinse with buffered water.
7. Differentiate for few seconds with glycerine-ether freshly diluted with four volumes of ethanol.
8. Dehydrate by a rapid dip in ethanol.
9. Clear in xylol.
10. Mount using mounting medium and a cover slip.

RESULT

Cytoplasm of immature cells is blue, that of erythroid cells is orange and of maturing and mature granulocytes is pale pink. Granules of eosinophils stain bright red.

EXAMINATION OF BIOPSY SECTIONS OF BONE MARROW

First scan whole of the section with scanner objective for relative distribution of cellular and fatty marrow. In normal adults this ratio is 1:2 to 1:1. Then examine for gross abnormalities like necrosis, granulomas, metastasis and lymphoid aggregates. Note any abnormal infiltrate and its location. Switch to x10 objective and note number and distribution of megakaryocytes. Also note the relative distribution of various haemopoietic elements. Switch to x40 objective and note the morphology of both normal and abnormal constituent cells. Examine for any parasites or other inclusions in the cell. Then examine the section stained with reticulin stain and note the amount of fibrosis. In a normal marrow only a few scattered fine fibres are seen whereas in myelofibrosis interlacing bundles of thick fibres are seen. The fibrosis in between can be graded from I to III, last being grade IV. If required May-Grunwald-Giemsa stained sections should be examined. These are ideal for differentiating between megaloblasts and other blasts as well as for identifying intracellular and extracellular parasites.

REPORTING OF BONE MARROW TREPHINE BIOPSY SECTIONS

Detailed description on reporting of bone marrow trephine biopsy sections is beyond the scope of this book. However a general outline is given below.

First gross appearance and size of the biopsy specimen is reported. Microscopic findings are reported in the same sequence as examined. All details should be clearly mentioned. Any abnormalities noted should be highlighted. Then the amount of fibrosis should be reported. Then give the conclusions drawn from the findings. Finally give the most likely diagnosis. This may be followed by suggestions regarding further investigations

37. BLOOD CELL CYTOCHEMISTRY

Cytochemical techniques can be applied to both red blood cells and white blood cells to demonstrate various chemical constituents, in the cell. These techniques are extremely useful in the diagnosis of various haematological disorders. Their main use however lies in the study of immature white cells (blasts) to classify various types of leukaemias and identification of maturation abnormalities in the myelodysplastic syndromes and myeloproliferative disorders. Cytochemical techniques are interpreted both at light microscopic and electron microscopic levels. Some techniques, however, can only be interpreted by the use of an electron microscope e.g., platelet peroxidase (PPO) activity in megakaryoblasts.

RED BLOOD CELL CYTOCHEMISTRY

Cytochemical techniques are applied to both developing and mature erythroid cells to demonstrate:

1. Iron incorporation defects: Perl's reaction for siderotic granules or haemosiderin.
2. Haemoglobin defects: HbF, HbH inclusions, Heinz bodies etc.
3. Enzyme defects: Demonstration of G6PD deficiency.

These methods are described in detail in the relevant chapters.

WHITE BLOOD CELL CYTOCHEMISTRY

Main use of cytochemistry in haematology involves leucocytes. It is used:

1. To differentiate between normal and abnormal neutrophils {Leucocyte/ Neutrophil Alkaline Phosphatase (LAP/NAP)} in order to differentiate between leukemoid reaction and Myeloproliferative disorder.
2. To study enzyme abnormalities of leucocytes.
3. To characterise cells in Lymphoproliferative disorders {Acid phosphatase (ACP), Tartrate resistant acid phosphatase (TRAP)}.
4. To study pattern of differentiation of early granulocytic and monocytic cells {Myeloperoxidase (MPO), Esterases etc.}

General precautions and instructions, applicable to all cytochemical staining procedures, are as under:

1. Top quality reagents should be used.
2. All glassware used must be washed with detergent and then with ample water thoroughly.
3. Blood or bone marrow smears should be prepared directly and not from the blood containing anticoagulant.

A positive control must be included with each batch of patient slides.

LEUCOCYTE/NEUTROPHIL ALKALINE PHOSPHATASE (LAP/NAP)

LAP activity is found predominantly in mature neutrophils, some activity in metamyelocytes and in reticulum cells of the bone marrow. It is associated with distinct tubular structures in the cytoplasm. It is allowed to react upon a conjugated substrate, Naphthol AS Phosphate, which produces an insoluble coloured compound localised to the site of activity of enzyme.

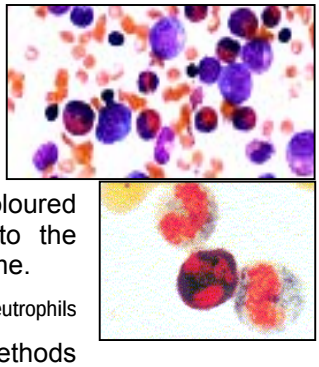


Figure 37.1: LAP activity in neutrophils

There are several methods but the method described by Rutenberg et al gives best results. However, it is seldom feasible to procure and use individual reagents because of limited workload. The reagents are available in kit form by several manufacturers. It is advisable to use kits. The method given in the literature enclosed within the kit should be followed. With each test or batch of test a positive control slide prepared from the blood obtained from a neonate or patient with acute infection must be stained. Blood films should be made soon after blood collection, preferably within 30 min, as LAP activity decreases rapidly in EDTA anticoagulated blood.

Result: Discrete bright blue granules represent sites of LAP activity.

Scoring: The LAP/NAP activity is represented as a score in absolute numbers. For purposes of scoring the activity is graded as under:

0	No granules at all
1	Very few granules
2	Few to moderately high number of granules
3	Moderately high to numerous granules
4	Cytoplasm packed with granules

For scoring the activity, 100 consecutive mature

neutrophils are graded for activity under high power/oil immersion lens of microscope. The score is the sum of individual scores of 100 neutrophils. The blood films should be made soon after blood collection as NAP activity decreases rapidly in EDTA anticoagulated blood.

Reference range

In neonates	150-300
In children and adults	35-100

Significance:

1. High scores are found in:
   - Leukemoid reaction (page 268)
   - Infections
   - Cirrhosis of liver
   - Polycythemia vera
   - Down's syndrome
   - Active Hodgkin's disease
   - Blast transformation in CGL
   - Aplastic anaemia
   - Physiological in newborn, children and pregnant females
2. Low scores are found in:
   - CGL
   - PNH

MYELOPEROXIDASE (MPO, POX)

Myeloperoxidase is an enzyme present in the azurophilic lysosomal granules of granulocytes and their precursor, in eosinophil granules and monocytes. In neutrophils these granules are larger and appear first i.e., in blast stage. In monocytes these are small and appear late. It is also present in the specific granules of eosinophils and basophils. The enzyme acts upon benzidine in the presence of hydrogen peroxide to yield a coloured product localised to the site of enzyme activity. As benzidine is a carcinogenic substance so the alternate substrates may also be used. The substrate of choice is 3,3'-diamin benzidine (DAB). Kits utilising this substrate are commercially available and are recommended for the laboratories, which have a large workload. For smaller workload method based on benzidine is cheap and easy. Method is described below in detail.

Reagents

Solution-I:

Benzidine base	2.0 g
Basic fuchsin	1.2 g
Sodium nitroprusside (saturated solution)	4 ml
Ethyl alcohol (95 percent)	400 ml

Grind 2.0 g of benzidine base in a mortar with a small amount of ethyl alcohol. Add the rest of the alcohol mixing well in the mortar. Filter this solution into a bottle. To the filtered solution add basic fuchsin and 3 ml of saturated solution of sodium nitroprusside. Age for 2-4 days. Keep at room temperature in dark dropping bottles. Add additional sodium nitroprusside if staining becomes less distinct.

Solution-II: Prepared fresh each time by adding 4 drops of 3% analytical grade hydrogen peroxide to 25 ml of distilled water.

Procedure

1. Cut filter paper to a size about half an inch longer than the size of the slide and place over the smear.
2. Drop solution-I onto the filter paper until it is just wet (approximately 8-10 drops). Let it stand for half to one min.
3. Flood the slide with solution-II. Gently blow to mix the two solutions. Let it stand for half to one min.
4. Peel off the filter paper. Smear should be of definite red colour. To remove excess of stain, hold the slide with forceps and wash in running water.
5. Counterstain with 1:10 diluted Giemsa's stain for 40 min.
6. Wash with tap water, dry and mount.

Result: Sites with enzyme activity stain pink to red.

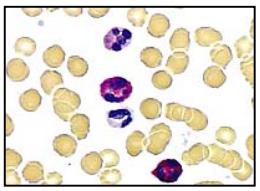


Figure 37.2: Myeloperoxidase stain

Significance: The activity is seen with increasing strength in all cells of granulocytic series except very early myeloblasts, which may be negative. Eosinophil granules stain strongly. Promonocytes and monocytes also show activity whereas monoblasts and all stages of lymphoid cells are negative.

SUDAN BLACK B (SBB) STAINING

It is a lipophilic dye that binds irreversibly to an unidentified granular component, most probably phospholipid membrane of granules in granulocytes, eosinophils and some monocytes containing MPO activity either directly or by an enzyme linked reaction. Reaction parallels MPO activity in various cells. Being simpler than MPO method it is preferred by most of the laboratories. FAB group for classification of leukaemias recommends it. The method of Sheehan and Storey has remained undisputed and is described below:

Reagents

1. Fixative: 40% Formaldehyde
2. Solution A (Stain): It is prepared by dissolving 0.3g of Sudan black-B in 100 ml

of absolute ethyl alcohol. The mixture is frequently shaken vigorously for 1-2 days to dissolve all the dye and then filtered.

3. Solution B (Buffer): 16 mg of pure phenol crystals are dissolved in 30 ml of absolute ethyl alcohol. Add it to 100 ml of 0.3% solution of disodium hydrogen phosphate in distilled water. Stir vigorously to dissolve the phenol and filter.
4. Sudan Black-B staining solution: 30 ml of solution A is mixed with 20 ml of solution B and filtered through double layer of filter paper. Mixture should be neutral or slightly alkaline.
5. Counterstain: Giemsa stain stock solution (as for staining of thick film for malarial parasite) is diluted 1/50 with distilled water.

Procedure

1. Fix air-dried smears in formalin vapour for 10 min. This is done by exposing smears to pure formalin in a jar so that formalin does not come in contact with the smear.
2. Immerse slides for I hour in SBB staining solution.
3. Transfer slides to a staining rack and immediately flood with 70% alcohol. After 30 seconds, tip the alcohol off and flood it again with 70% alcohol for 30 seconds, and repeat it three times.
4. Counterstain with diluted Giemsa's stain for 40 min.
5. Wash, air dry and mount.

Result: The granules stain grey to black.

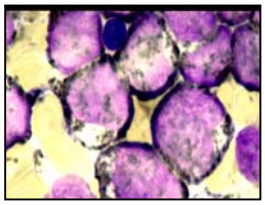


Figure 37.3: Sudan black stain

Interpretation: As for MPO. The only notable difference is in the eosinophil granules, which have a clear core when stained with SBB.

ACID PHOSPHATASE (ACP) STAINING

The activity of enzyme ACP is present in almost all haemopoietic cells. However these cells differ in quantity and distribution of this hydrolase in the cell. These differences are utilised in the differential diagnosis of malignant disorders of haemopoietic cells. Like other enzymes, its activity is also demonstrated by conversion of a colourless substrate to a stable coloured compound visible under light microscope.

Reagents and Procedure

At least 9 different chemicals are required to prepare the reagents in the laboratory. Some of these are very expensive and may not be easily available. For low workload laboratories, therefore, preparation of reagents in the laboratory may not be cost effective. All the reagents are available commercially in the form of kit. It is advisable to procure the kit and follow the procedure recommended by the manufacturer.

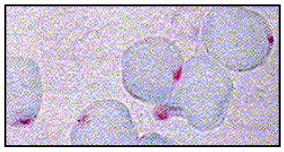


Figure 37.4: Acid phosphatase stain

Result

If the kit utilises Naphthol-AS-BI phosphate as substrate, which is the commonly used substrate, then the ACP activity is revealed by bright red granules. Otherwise results are indicated in the method sheet by the manufacturer.

Significance

Granulocytes are strongly positive. In bone marrow, macrophages, plasma cells and megakaryocytes are strongly positive. Monoblasts react more strongly than Myeloblasts. T-lymphocytes of all stages show ACP activity. In T-ALL the reaction is localised to an area corresponding to Golgi zone (polar). The reaction is also positive in T-CLL but not so consistently. About two third cases of T-PLL also show the activity. In all these, reaction is inhibited by prior treatment with tartrate. In Hairy cell leukaemia the reaction is not inhibited by tartrate and hence called **Tartrate Resistant Acid Phosphatase (TRAP)**. Some B-Prolymphocytes may also show a weak positive reaction, which may also be resistant to tartrate.

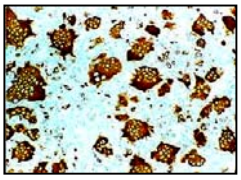


Figure 37.5: Acid phosphatase (TRAP) stain

PERIODIC ACID-SCHIFF REACTION (PAS)

Glycogen is the stored energy source for several cells in the body. It is present in almost all cells of haemopoietic tissue. Its quantity and distribution inside various haemopoietic cells is however different. These differences are utilised to differentiate between various types of cells. Glycogen is a carbohydrate and reacts positively in PAS reaction. It is differentiated from other carbohydrates by the fact that when treated with diastase, the reaction becomes negative. In this reaction carbohydrate is liberated from the protein and is oxidised to aldehyde by Schiff reagent. These are coloured pink in subsequent reaction.

Reagents

More than 10 different chemicals are required to

prepare the reagents in the laboratory. Some of these are very expensive and may not be easily available. For low workload laboratories, therefore, preparation of reagents in the laboratory may not be cost effective. All the reagents are available commercially in the form of kit. It is advisable to procure the kit and follow the procedure recommended by the manufacturer.

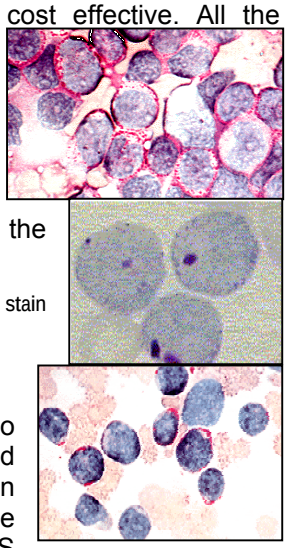


Figure 37.6: PAS stain

Result

Glycogen stains pink to bright red in untreated smear but reaction disappears in diastase treated smear. Other PAS positive materials give positive reaction in both treated and untreated smears.

Interpretation

The cytoplasmic positivity may be diffuse or granular. Diffuse positive reaction with few granules is seen in myeloblasts and monoblasts. Negative reaction is seen in normal erythroblasts. Neutrophils react most strongly whereas specific granules of eosinophils are negative with diffuse cytoplasmic positivity. Megakaryocytic cells, and platelets are positive. In common type of childhood ALL (C-ALL) blasts may contain blocks of PAS positive material. Cells of chronic B-lymphoproliferative disorders often have increased number of positive granules. Erythroblasts in almost all diseased states stain diffuse pink, whereas in AML-M6 there may be large blocks of PAS positive material in the cytoplasm.

ESTERASES

These are group of 9 (1-9) hydrolases, best demonstrated by Naphthol AS-D Chloroacetate as substrate. These are called **specific** esterases and are not inhibited by sodium fluoride.

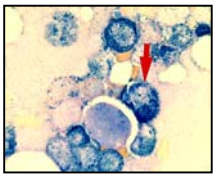


Figure 37.7

The remaining are inhibited by sodium fluoride and are called **non-specific** esterases (NSE). These are identified by the name of substrate used to demonstrate them. All important esterase stains are commercially available in the form of kits.

Chloroacetate Esterase (CAE)

It is a specific esterase present in granulocytes and mast cells. The cytoplasmic CAE activity appears as myeloblasts mature to promyelocytes. Promyelocytes and myelocytes stain strongly. The enzyme is optimally active at *p*H 7.0-7.6 and it is not inhibited by sodium fluoride. It parallels that of MPO or SBB. However, it is usually negative in monoblasts. It is used in combination with ANAE in demonstrating Monocytic and Granulocytic precursors in the same preparation.

α Naphthol Acetate Esterase (ANAE)

The reaction produced is diffuse red or brown in colour. This hydrolase gives a distinct positive reaction in normal and leukaemic monocytic cells and T lineage lymphoid cells. In monocytes the reaction is diffuse and is sensitive to sodium fluoride. Whereas in T-lymphoid cells it is localised as a dot and is resistant to sodium fluoride. Megakaryocytes stain strongly and leukaemic megakaryocytes may show focal and diffuse positivity.

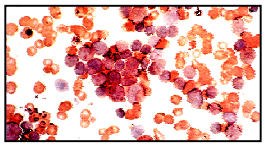


Figure 37.8: ANAE stain

Leukaemic erythroblasts may show focal or diffuse positivity. Its value lies in:

1. The differentiation of M1 from M5
2. Diagnosis of M6 and M7 in which blasts give positive reaction localised to Golgi area. The reaction is sensitive to fluoride.
3. Diagnosis of T-ALL. Localised reaction is resistant to fluoride.
4. To differentiate between T-PLL and B-PLL.

OIL RED O STAIN

Purpose: To stain fat present in the cells.
Principle: Oil red O is soluble in fat and thus stains it orange to red.

Requirements

1. Oil red O Solution: It is prepared by dissolving 2 g Oil red O stain in 50 ml 70% Alcohol and 50 ml Acetone.
2. Glycerine Jelly: It is prepared by dissolving, with the help of heat, 10 g gelatin in 60 ml distilled water. To it is added 70 ml Glycerine and 1 ml Phenol.

Procedure

1. Dip section in 70% alcohol for only a second.
2. Place in oil red O in a tightly closed container for 5 min.
3. Wash quickly in 70% alcohol. Avoid folding

of section.
4. Wash in water.
5. Counter stain in Harris's haematoxylin for a few seconds.
6. Wash in water.
7. Blue in ammonia water.
8. Wash in water.
9. Mount in glycerine jelly.

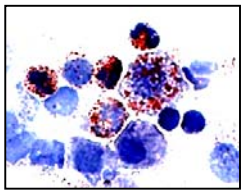


Figure 37.9: Oil red O stain

Result

Fat: Orange to red; Nuclei: Blue

Table 37.1: Differential staining characteristics in Acute Lymphoblastic Leukaemia

REACTION	EARLY B-ALL	C-ALL	T-ALL	B-ALL
POX/SBB	-	-	-	-
PAS	- to ++ Coarse granular	+ to ++ Coarse granular	-/+	-
ACP	-/+	-/+	++ to +++	-
ANAE	-/+	-/+	++ Localised NaFl R	-
OIL RED O	-	-	-	+

Table 37.2: Differential staining characteristics in Acute Myeloid (Non-Lymphoblastic) Leukaemia

REACTION	M1	M2	M3	M4	M5	M6	M7
POX SBB CAE	+ to ++ >3% blasts	++	+++	+ to ++	-/+	+ In myeloblast	-
ANAE	-	+/-	- to +	+ to ++ NaFl S	+++	+ Localised NaFl S	++ Localised NaFl S
NASDA	+	+	++	+ to ++ NaFl S	+++ NaFl S	++ NaFl S	-
ACP	-/+	+	+ to ++	+ to ++	+++	+/-	++
PAS	+ Diffuse	+ Diffuse	++ Diffuse	+ to ++	+ to ++	+	+ to ++

38. HAEMOGLOBIN DISORDERS

Haemoglobin is the oxygen carrying pigment of the red blood cells. It is a conjugated protein composed of four subunits. Each subunit is composed of a globin chain and haem group. Each haem group has a single iron atom in the form of ferrous ion. When red blood cells pass through the lungs, they take up oxygen from the air, which combines with the ferrous iron of haem. This reaction is not that of oxidation, but of oxygenation i.e., the ferrous form of iron is not converted to the ferric form. Since there are four

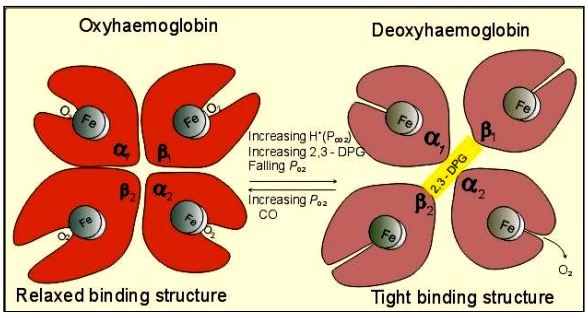
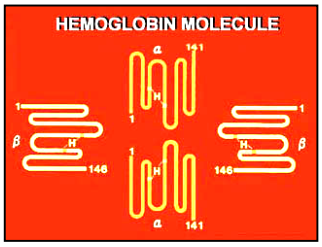


Figure 38.1: Structure and function of haemoglobin

haem groups in one molecule of haemoglobin, it can combine with four oxygen molecules. There are various types of haemoglobins that differ from each other with respect to the structure of their globin chains. The haem moiety is identical in all types of haemoglobins. The α-globin chain consists of 141 amino acids, whereas, β-chain is composed of 146 amino acids. The haemoglobins consist of 2 α- and 2 non-α chains. Composition of various haemoglobins is shown in Table 38.1. Foetal haemoglobin (HbF) is the predominant haemoglobin in the intrauterine life. At birth 90% Hb is HbF. After birth HbF starts decreasing and is replaced with HbA and HbA_2. At six months it is about 5%, and the adult level of 1% is reached at the end of first year of life. In adults haemoglobin consists of 97% HbA and 3% HbA_2.



CLASSIFICATION OF HAEMOGLOBIN DISORDERS

These are broadly classified into quantitative and qualitative disorders.

1. **Quantitative disorders**: In these there is reduced synthesis of a structurally normal globin chain. These are called **Thalassaemias** and are named after the deficient globin chain. For example in β-thalassaemia there is reduced synthesis of β globin chains.
2. **Qualitative disorders**: In this category the globin chain being synthesised is structurally abnormal. This is due to the substitution of one or more normal amino acids in any of the globin chains with different amino acids. In sickle cell anaemia, valine substitutes glutamic acid at the sixth position of the β chain.

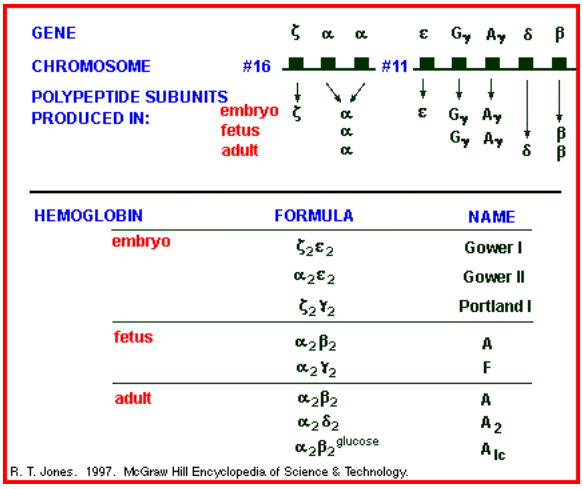
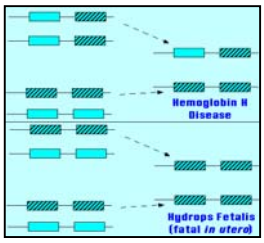


Table 38.1: Haemoglobin genes and variants

QUANTITATIVE DISORDERS OF HAEMOGLOBIN SYNTHESIS (THALASSAEMIAS)



Thalassaemias are inherited quantitative disorders of globin chain synthesis. These are classified on the basis of deficient or absent synthesis of the chains involved. The following are the main types of thalassaemias.

1. **α-thalassaemias**: There is deficient or absent synthesis of α globin chains.
   a. α-thalassaemia silent carrier state (- α/ αα)
   b. α-thalassaemia trait (- α/- α or - -/αα)
   c. HbH disease (- -/- α)
   d. Hb Barts (hydrops foetalis syndrome)(- -/- -)
2. **β-thalassaemias**: There is deficient or no synthesis of β globin chains.
   a. β-thalassaemia trait – ($\beta^{+/o}$/ β)
   b. β-thalassaemia major – ($\beta^{+/o}$/ $\beta^{+/o}$)
   c. Thalassaemia intermedia – (variable)
3. **δβ-thalassaemia**: There is deficient synthesis of both δ and β globin chains.

QUALITATIVE OR STRUCTURAL DISORDERS OF HAEMOGLOBIN

Structural disorders are further classified on the basis of physical and chemical properties of abnormal Hb molecule into:

1. Haemoglobins with altered solubility (HbS, C etc.).
2. Unstable haemoglobins.
3. Haemoglobins with altered oxygen affinity.
4. Thalassaemic structural variants (Hb Lepore, HbE, Hb Constant Spring)

MISCELLANEOUS HAEMOGLOBIN ABNORMALITIES

Some haemoglobins are neither structurally nor functionally abnormal and have little clinical implication. Example is Hereditary Persistence of Foetal Haemoglobin (HPFH) where HbF persists into adult life.

INVESTIGATIONS OF HAEMOGLOBIN DISORDERS

Following plan of investigations is suggested in a clinically suspected case of haemoglobin disorder:

1. Basic tests
   a. Full blood count.
   b. Red cell morphology
   c. Reticulocyte count
2. First line identification tests
   a. Hb electrophoresis on cellulose acetate membrane
3. Second line identification tests (based on results of Hb electrophoresis on cellulose acetate membrane)
   a. Estimation of HbA_2
   b. Estimation of HbF
   c. Test for sickling
4. Other tests
   a. PCR for identification of mutations.
   b. Electrophoresis on other media like agar gel, starch gel etc., in various buffers.
   c. Tests for Unstable Hb
   d. Tests for Methaemoglobin
   e. Tests for altered affinity haemoglobin
   f. Isoelectric focusing
   g. Estimation of rate of globin chain synthesis.

Only some of these are done in a routine laboratory.

HAEMOGLOBIN ELECTROPHORESIS

For general principles and procedure see section on ELECTROPHORESIS on page 38. Cellulose acetate membrane is used for initial haemoglobin electrophoresis. It is a smooth, homogeneous and strong medium on which separation of different types of haemoglobins is excellent. For more precise results, polyacrylamide gel, starch gel and agar gel are used. Before proceeding for haemoglobin electrophoresis it is necessary to prepare the haemolysate i.e., to break the red cells so that haemoglobin comes out of the cells.

Preparation of haemolysate

1. Any anticoagulant may be used but EDTA is suitable for this purpose.
2. About 2 ml of anticoagulated blood is taken and three washings are given with isotonic saline. This is done by adding normal saline 4 times of blood volume, mixing, centrifuging and decanting supernatant.
3. After the final wash, add half volume of distilled water to the packed cells left behind and shake. This will cause haemolysis of the cells.
4. Add equal volume of carbon tetrachloride and mix well.
5. Centrifuge the mixture at about 3000-RPM for 15 min. The clear red lysate is pipetted off in another test tube.
6. Lysate can be stored at 4°C if not immediately used and can be transported to another laboratory (in ice), if facilities are not available.
7. The Hb in the lysate should be about 10 g/dl. If it is more than that, add distilled water to adjust the required haemoglobin concentration using formula given on page 48:

Example:

Hb of lysate= 15 g/dl
Volume of lysate= 0.5 ml
Required Hb of lysate= 10 g/dl

$V_1C_1 = V_2C_2$

$$V_2 = \frac{V_1 \times C_1}{C_2} = \frac{0.5 \text{ x } 15}{10} = 0.75 \text{ ml}$$

Volume of distilled water to be added
= 0.75-0.5
= 0.25 ml

Cellulose acetate membrane electrophoresis
Various buffers can be used for haemoglobin electrophoresis at different pH, using cellulose acetate membrane strips.

Requirements
1. Tris-EDTA-Borate buffer *p*H 7.9. It is the buffer for routine haemoglobin studies and is prepared as under:

Boric acid	6.4 g
Tris aminomethane	5.1 g
Disodium EDTA	0.3 g
Water to make	1 litre

2. Tris-EDTA-Borate buffer *p*H 8.9. It is the buffer for HbA_2 estimation and is prepared as under:

Tris aminomethane	14.4 g
Disodium EDTA	1.56 g
Boric acid	0.92 g
Water to make	1 litre

3. Electrophoresis apparatus (page 38)
4. Cellulose acetate membrane strips
5. Trichloracetic acid 3%
6. Ponceau S stain 0.2%
7. Acetic acid 5%
8. Staining trays
9. Scissors

Procedure
Follow the procedure given in the section on ELECTROPHORESIS on page 38 with following modifications:
1. Apply the lysate near the cathode bridge towards the right of the base line using a capillary tube.
2. Run at 200 V for 30-45 min.
3. After staining the strip, dry it between two layers of filter paper and then in an incubator at 37°C.
4. It is necessary to run a normal sample and positive controls each time for comparison.

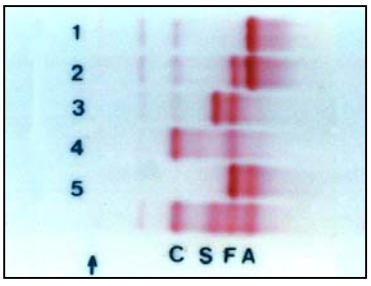


Figure 38.2: Electrophoretic mobility of haemoglobins

Result
The relative electrophoretic mobility of different Haemoglobins is shown in Figure 38.2: Electrophoretic mobility of haemoglobins.

ESTIMATION OF HbA_2

HbA_2 can be estimated chromatographically using columns or by electrophoresis. HbA_2 columns are available in kit form. This is more accurate method but is expensive unless the columns are prepared in house. For quantitation by electrophoresis, haemolysate is electrophoresed on cellulose acetate membrane using Tris-EDTA borate buffer of *p*H 8.9. Follow the steps in the above procedure. HbA_2 can be quantitated by reading the density of its band on the strip in a densitometer. Alternatively, it can be quantitated by eluting and reading its absorbance in a suitable colorimeter or spectrophotometer. Method for the later procedure is as follows:

Requirements
1. Electrophoresed strip of Hb
2. Tris EDTA borate buffer of *p*H 8.9
3. Test tubes
4. Pipettes
5. Spectrophotometer

Procedure
1. Set up 3 tubes marked A, A_2 and Blank (B).
2. Put 20 ml buffer in tube A and 4 ml in each of tubes A_2 and B.
3. Cleanly cut portions of strip bearing HbA and HbA_2 bands.
4. Place the cut portions of strip of HbA in tube A, of HbA_2 in tube A_2 and a piece of clear strip in tube B.
5. Allow to elute for 30 min.
6. Read absorbance of tubes A and A_2 against B in a spectrophotometer at 416 nm.

Calculation

$$\%\ HbA2 = \frac{\text{Abs } HbA_2}{\text{Abs } HbA_2 + (\text{Abs } HbA \times 5)} \times 100$$

Reference range: 1.5-3.5%

Interpretation
HbA_2 of >3.5% is diagnostic of β thalassaemia trait. If iron deficiency coexists along with β thalassaemia trait then readings between 3.0% and 3.5% may be seen. In such cases HbA_2 estimation is repeated after correction of iron deficiency.

ESTIMATION OF HbF

HbF can be estimated qualitatively by staining *in situ*. This is done by the acid elution technique. Or it is estimated quantitatively by alkali denaturation method. Both of these procedures are described below.

ACID ELUTION METHOD (KLEIHAUER'S TEST)

Principle

The test is based on the principle that HbF resists acid elution to a greater extent than HbA. It is performed on smears of blood made on a glass slide. Cells, which contain HbA are cleared of their haemoglobin whereas cells, which contain HbF, retain the haemoglobin and hence stain pink.

Requirements

1. Fixative: 80 % ethyl alcohol
2. Elution solution
   a. Solution A: Dissolve 7.5 g haematoxylin in one litre of 90 % ethanol.
   b. Solution B: Dissolve 24 g ferric chloride in 20 ml of 2.5 mol/L HCl and make the volume to one litre with distilled water.
   c. Working solution: Mix 5 volumes of solution A and 1 volume of solution B. The *p*H should be 1.5. The solution is stored at 4°C. It is important to filter the solution before use otherwise a deposit is left on the stained slides.
3. Counter stain: Dissolve 2.5 g eosin in one litre of distilled water.

Procedure

1. Prepare peripheral blood smears from EDTA anticoagulated blood.
2. Fix the slides in 80 % ethanol for 5 min.
3. Remove the slides from the fixative and wash in running tap water.
4. Dry the slides in air.
5. Place the slides in elution solution for 20 seconds. *p*H and time is absolutely critical.
6. Rinse immediately in tap water.
7. Dry in air again.
8. Counter stain with aqueous eosin for 5 min.
9. Dry the slides after rinsing in tap water.
10. See under oil immersion lens.

Result

Cells containing HbF stain pink, whereas the cells that contain HbA appear as clear ghost cells.

SEMI-QUANTITATIVE ESTIMATION OF FOETAL BLOOD IN THE MATERNAL CIRCULATION

Kleihauer test can also be used to find out roughly the volume of foetal blood entering the maternal circulation. This is important because in feto-maternal incompatibility the dose of anti-Rh D immunoglobulin given to the mother depends upon the quantity of blood that has entered the maternal circulation. If the loss is less than 4 ml, then the usual dosage (100 µg) is enough to prevent the mother from sensitisation. Otherwise dose is to be increased. The procedure is as follows:

1. Prepare thin, uniform smears of maternal blood. The cells should be separate and uniformly spread.
2. Stain as detailed above.
3. Focus the stained film under low power.
4. Count the number of foetal cells (darkly staining) per low power field and also count the number of adult red cells (ghost cells).
5. The volume (ml) of foetal red cells in the maternal circulation can be calculated by the following formula:

$$\frac{2000 \times \text{Foetal red cells} \times 1.33}{\text{Adult red cells}}$$

Where 2000 is the approximate maternal red cells in 1 ml blood, 1.33 is the correction factor (because all the foetal red cells do not retain their haemoglobin after acid elution). If, however, there are less than 10 foetal red cells in 5 low power fields, then it can be safely assumed that less than 4 ml of foetal blood has crossed the placental barrier.

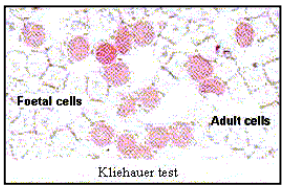


Figure 38.3: Kleihauer test

ESTIMATION OF HbF - BETKE'S METHOD

Principle

HbF is more resistant than HbA to denaturation by an alkaline solution of NaOH. This method detects HbF in the range of 0.5-50%.

Reagents

1. Haemolysate
2. Saturated ammonium sulphate solution
3. Sodium hydroxide 1.2 mol/L
4. Drabkin's solution
5. Pipettes
6. Test tubes and test tube stand
7. Filter paper
8. Spectrophotometer

Procedure

1. Prepare a cyanmethaemoglobin (HiCN) solution by adding 0.2 ml of haemolysate to 4 ml Drabkin's solution.
2. Take two test tubes and label them as test and standard. Place these in a stand.
3. In test tube marked test add following:
   a. Hi CN 2.8 ml
   b. Na OH 0.2 ml
   (Wait for 2 min)

c. Saturated ammonium sulphate 2.0 ml
4. Mix and wait for 10 min.
5. After mixing thoroughly, filter the solution.
6. Make a 25% solution of standard by adding 0.7 ml of HiCN solution to 4.3 ml of Drabkin's solution in a test tube marked standard.
7. Read against distilled water the absorbance of both at 540 nm.

Calculation

$$\% \text{ HbF} = \frac{\text{Abs test}}{\text{Abs Std}} \times 25$$

ESTIMATION OF HbF - SINGER'S METHOD

Principle
HbF is more resistant than HbA to denaturation by an alkaline solution of NaOH. This method detects HbF over 50% as well.

Reagents
1. Haemolysate
2. Sodium hydroxide 1.2 mol/L
3. Acidified 50% saturated ammonium sulphate (50% of saturated ammonium sulphate 800 ml, 10N HCl 2 ml)
4. Ammonia 0.04% V/V

Procedure
1. Take two test tubes and mark them as test and standard.
2. To the tube marked test, add 3.2 ml of lysate and 0.2 ml sodium hydroxide.
3. Shake the mixture vigorously and start a stopwatch.
4. After exactly one min add 6.6 ml of acidified 50% saturated ammonium sulphate.
5. Shake vigorously and filter.
6. To the tube labelled as standard add 0.2 ml of original haemolysate and 4.8 ml of 0.04% (v/v) ammonia solution.
7. Read absorbance of both against distilled water at 540 nm.

Calculation

$$\text{Hb F\%} = \frac{\text{Abs Test}}{\text{Abs Std}} \times 100$$

Reference Range
After one year age <1%

DEMONSTRATION OF HbH INCLUSIONS

Haemoglobin H (β 4) is formed in red cells of patients with α-thalassaemia. It should be suspected when a patient has red cell indices suggestive of thalassaemia namely a low MCV, MCH and high red cell count, but does not have a raised HbA_2 or HbF and is not iron deficient.

Principle
Red cells containing HbH when exposed to supra vital stains e.g., brilliant cresyl blue as in reticulocyte preparations, form multiple blue green dots inside the red cells, giving pitted golf ball appearance.

Requirements
1. Brilliant cresyl blue 10 g/L in citrate saline (page 255).
2. Glass slides, Pasteur pipette
3. Test tube
4. Glass slides
5. Microscope

Procedure
1. Mix equal volumes of brilliant cresyl blue solution and EDTA anticoagulated blood.
2. Incubate at 37°C for 2 hours (better in a water bath).
3. Make films and allow to dry.
4. See under oil immersion lens for typical HbH inclusions. HbH precipitates as multiple pale staining greenish blue, almost spherical bodies of varying sizes. They can be clearly differentiated from the darker staining reticulo-filamentous material of reticulocytes. They typically have a golf ball appearance.

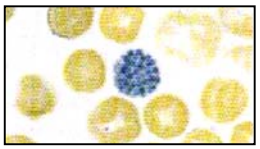


Figure 38.4: Hb-H inclusions

Precautions
Hb-H is an unstable Hb, therefore, fresh blood should be used for demonstration of Hb-H inclusions.

Interpretation
HbH inclusions are diagnostic of α thalassaemia. The number of cells containing HbH inclusions varies according to the type of α thalassaemia. In α thalassaemia trait, 0.01-1% red cells contain inclusions. In HbH disease at least 10% of the red cells contain the inclusions.

DETECTION OF SICKLE HAEMOGLOBIN (HbS)

HbS is found in sickle cell disease. In this abnormal Hb, valine is substituted for glutamic acid at the sixth position of the β-globin chain. One of the properties of HbS, which is responsible for the clinical symptoms, is its conversion into insoluble crystals when exposed to low oxygen tension. Tubular filaments are produced and the red cells become sickle shaped. HbS can be detected by the qualitative solubility test, sickling test and haemoglobin electrophoresis. Hb electrophoresis has been described earlier while the other two tests are

described below.

QUALITATIVE SOLUBILITY TEST

Principle
The test is based on the principle that HbS is relatively insoluble in concentrated phosphate buffer in the presence of reducing substances.

Requirements
1. Phosphate buffer, *p*H 7.1
   a. Potassium dihydrogen phosphate 33.78 g
   b. Dipotassium hydrogen phosphate 59.33 g
   c. Saponin 2.5 g
   d. Water 250 ml
2. Dissolve 0.1 g of sodium metabisulphite in 10 ml of buffer, prior to use, to make working solution.

Procedure
1. Take 2 ml of the working solution and add 4 drops of EDTA anticoagulated whole blood to it. Mix thoroughly.
2. Centrifuge at 1200 g for 5 min.
3. Remove the tube and note the appearance of the solution.

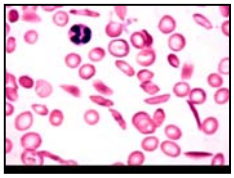


Figure 38.5: Sickle cells

Interpretation
HbA is soluble in concentrated phosphate buffer, hence it gives a uniform red colour without any precipitate. If there is no HbA, and whole of the Hb is HbS, then only a red precipitate will be formed, whereas rest of the fluid will be clear. In cases of sickle cell trait, both a homogeneous red solution of HbA as well as a precipitate of HbS will be seen.

SICKLING TEST

Principle
This test is based on the decreased solubility of HbS at low oxygen tension. For this purpose a reducing reagent e.g., sodium dithionite or sodium metabisulphite is added or oxygen is excluded by sealing the blood under a cover slip.

Requirements
1. Sodium metabisulphite 2%: Dissolve 2 g in 100 ml distilled water.
2. Glass slides
3. Cover slips
4. Bunsen burner
5. White petroleum jelly, or wax
6. Microscope

Procedure[1]
1. Add 5 drops of sodium metabisulphite to one drop of EDTA anticoagulated blood in a test tube and mix.
2. Put one drop from the mixture on a slide.
3. Place a cover slip on the mixture.
4. Take some wax or petroleum jelly on an iron rod and soften it by heating over flame of Bunsen burner. Apply the jelly on the sides of the cover slip so that no air can enter through it.
5. After sealing completely, look for sickling immediately and after 1-2 hours and after 12 hours.

Interpretation
Immediate sickling indicates HbS disease. Sickling after 1-2 hours and sometimes after 12 hours is suggestive of HbS trait.

DEMONSTRATION OF HEINZ BODIES

Heinz bodies are precipitated globin chains, which may be seen as red cell inclusions.

Principle
Heinz bodies are demonstrated by cytochemical staining but can also be seen in unstained preparations as refractile objects by lowering the microscope condenser, dark ground illumination or by phase contrast microscopy.

Requirements
- Methyl violet: Dissolve 0.5 g methyl violet in 100 ml 9 g/L NaCl and filter.
- Pipettes
- Test tube
- Glass slides
- Microscope

Procedure
- Mix 1 drop of EDTA anticoagulated blood and 4 drops of methyl violet solution in a test tube.
- Allow to stand for 10 min at room temperature.
- Prepare the films and allow to dry.
- See under oil immersion lens.

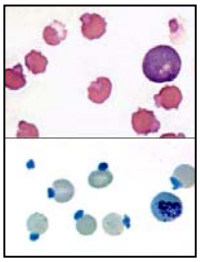


Figure 38.6: Heinz bodies

Interpretation
Methyl violet stains the Heinz bodies as intense purple inclusions in RBC. Their size varies from

[1] Test can also be performed without sodium metabisulphite but quality of sickling may not be good, particularly in Hb S trait.

1-3 µm. One or more may be present in a single cell, usually lying close to the cell membrane. The presence of Heinz bodies in the blood is a sign of chemical poisoning, drug intoxication, G6PD deficiency or the presence of an unstable haemoglobin e.g., Hb Koln. These may also be produced by the action on red cells of some aromatic nitro and amino compounds such as inorganic oxidising agents.

HEAT INSTABILITY TEST

Principle

When haemolysate is exposed to heat under controlled conditions unstable haemoglobins precipitate while normal Hb does not.

Requirements

1. Tris-HCl buffer, *p*H 7.4 (0.05 mol/L)
   - Tris 18.17 g
   - HCl 1 mol/L 42 ml

   Dissolve Tris in about 500 ml distilled water. Add HCl and make the volume to one litre with distilled water.
2. Drabkin's reagent
3. Pipettes
4. Test tubes and stand
5. Water bath at 50°C
6. Centrifuge
7. Spectrophotometer

Procedure

- Take two test tubes, mark test and standard.
- Wash red cells from freshly taken blood from patient and a normal control. Cells are washed 3 times in saline and then packed.
- Take 1 ml packed cells of patient in test tube marked test and 1 ml of control packed cells in test tube marked standard.
- Add 5 ml distilled water to both and shake vigorously to lyse.
- Add 5 ml buffer and mix.
- Centrifuge at 1200 g for 20 min and remove the stroma with pipette.
- Take another set of similarly marked test tubes and transfer 5 ml of treated lysate from each tube to corresponding new tube.
- Place both tubes in water bath at 50°C for one hour. Examine periodically for turbidity and flocculation. If test sample contains unstable haemoglobin then precipitate is formed. Control tube should remain clear.
- If precipitate is formed then centrifuge the tubes and transfer 1 ml clear supernatant to another tube.
- Take in another tube 1 ml lysate from step 7 (unheated).
- Add 19 ml Drabkin's reagent to both.
- Read absorbance of both at 280 nm.

Calculation

$$\% \text{ Unstable Hb} = \frac{\text{Abs sample - Abs heated sample}}{\text{Abs unheated sample}} \times 100$$

ISOPROPANOL PRECIPITATION TEST

Principle

When a lysate containing unstable haemoglobin is incubated in presence of Isopropanol, the unstable haemoglobin precipitates while the normal Hb does not.

Requirements

1. Isopropanol buffer
   - Tris 12.12 g
   - HCl 1 mol/L 42 ml
   - Distilled water 1 L
   - Isopropanol 170 ml

   Prepare Tris-HCl 0.1 mol/L buffer of *p*H 7.4 by dissolving Tris and HCl in distilled water making the volume to one litre. Take 830 ml of this buffer and add to it 170 ml Isopropanol (17% v/v). Store at 4°C.
2. Pipettes
3. Test tubes with stand
4. Centrifuge

Procedure

- Wash test RBC and control RBC three times in normal saline and pack by centrifugation as described earlier.
- Take 1 ml of each of packed cells in two tubes marked test and control. Add 1.5 ml distilled water to both and shake vigorously.
- Centrifuge at 1200 g for 20 min and remove stroma by a pipette.
- Take two tubes marked test and control and place 2 ml buffer into each. Place these in water bath at 37°C to warm for 5 min.
- Add 0.2 ml of test and control lysate to corresponding tubes. Stopper the tubes and mix by inversion.
- Replace in water bath and examine at 5, 20 and 30 min. In a positive test precipitate will appear in patient sample tube in 5 min becoming flocculant in 20 min. Control tube remains clear.

39. ENZYMOPATHIES AND MEMBRANOPATHIES

ENZYMOPATHIES

Like other cells of the body red blood cells also contain a number of enzymes for its metabolic processes. However, red blood cells differ from other cells of the body in that all the enzymes required throughout its life are produced before extrusion of nucleus and these decay with age of the cell, finally resulting in the death of RBC. Main metabolic pathways for which enzymes are required are:

- Anaerobic glycolytic pathway for energy production also called **Embden Meyerhof pathway**.
- **Pentose phosphate pathway**, which is aerobic and is utilised for maintenance of **reduced glutathione** to overcome oxidant stress.
- Trios phosphate pathway
- Purine metabolic pathway
- Pathway for degradation of RNA (pyrimidine)

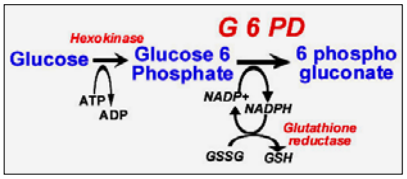
A number of enzymes are involved in these metabolic pathways. Quantitative or qualitative defects of these enzymes result in early death of RBCs under certain circumstances. These abnormalities are collectively called **enzymopathies**. Abnormalities of almost all known enzymes are described but majority of these are very rare occurring only in one in 10,000 or more individuals. Most commonly occurring enzymopathies of clinical significance are:

- Glucose-6-phosphate Dehydrogenase (G-6-PD) deficiency
- Pyruvate Kinase (PK) deficiency
- Pyrimidine-5-Nucleotidase (P-5ND) deficiency

The following paragraphs describe tests for detection of these enzymopathies.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY

Glucose-6-Phosphate dehydrogenase (G-6-PD) is an enzyme, which takes part in the hexose monophosphate shunt. It is required for production of NADPH to keep glutathione in reduced state. Metabolic pathways of red blood cell are shown here. Reduced glutathione is important in bearing the brunt of the oxidative stress. A small amount of met Hb (Hi) is produced all the time is converted back to Hb by reduced glutathione (GSH). Increased oxidant stress not only increases Hi production but also results in oxidation of many other components particularly of cell membrane. This results from:



1. Infections
2. Drugs:
   - Antimalarials e.g., chloroquine, quinine
   - Sulphonamides
   - Nitrofurans
   - Water soluble vitamin K
3. Foods like fava beans

There are two types of G-6-PD enzymes, B and A. **G-6-PD A** is only found in Africans. Abnormal variant of this type is A- but is not very severe. Most common type is **G-6-PD B** of which several variants have been described. These may produce either qualitative or quantitative or a mixed abnormality of the enzyme which may result in one of the following four clinical conditions:

- Congenital Nonspherocytic Haemolytic Anaemia
- Neonatal Jaundice
- Favism
- Acute Intravascular Haemolysis

G-6-PD SCREENING TESTS

Principle

G-6-PD is released from the lysed erythrocytes and catalyses the conversion of glucose-6-phosphate to 6-phosphogluconate with conversion of NADP to NADPH. Production of NADPH can be detected by:

- Its property to fluoresce in UV light.
- Conversion of Hi to Hb.
- Decolourisation of a reducible dye.

DYE REDUCTION TEST

In this test NADPH, in the presence of phenazine methosulphate (PMS), reduces the blue dye (dichlorophenol indophenol) to a colourless form. The rate at which the colour disappears in the reaction mixture is proportional

to the amount of G-6-PD in the red cells. Reagents are difficult to prepare in a routine laboratory. The test is available in kit form designed for single test use. Procedure may differ for each kit and is provided with the kit. Procedure and instructions given by the manufacturer of the kit should be strictly adhered to for good results.

METHAEMOGLOBIN REDUCTION TEST

Requirements

- Sodium nitrite-Glucose solution
  - Sodium nitrite 1.25 g
  - Glucose 5.0 g
  - Distilled water to 100 ml
- Methylene blue
  - Methylene blue chloride $3H_2O$ 0.15 g
  - Distilled water to 1 L
- Test tubes with stand
- Incubator/water bath
- Spectrophotometer

Procedure

1. Better to take blood in ACD and use it immediately. However, if test is put up immediately even EDTA anticoagulated blood can be used.
2. Adjust PCV to 0.4-0.5, removing enough plasma.
3. Take 3 test tubes and mark as Test (1), Positive Reference (2) and Normal reference (3).

	Tube (1)	Tube (2)	Tube (3)
Reagent-1	0.1 ml	0.1 ml	-
Reagent-2	0.1 ml	-	-
Test blood	2 ml	-	-
Normal blood	-	2 ml	2 ml

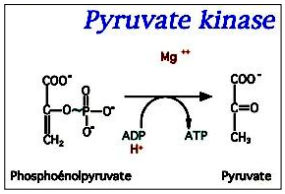
   (These tubes can be used fresh or can be evaporated to dryness and stored at 4°C for 6 months for future use)
   a. Mix well by inversion.
4. Incubate at 37°C for 3 hours without shaking.
5. Dilute 0.1 ml from each tube with 10 ml distilled water and compare visually after 2-10 min. Normal reference (tube-3) remains clear red. Positive reference (tube-2) becomes brown. Positive test will give shades of brown depending upon the severity of the deficiency.

QUANTITATIVE ESTIMATION OF G-6-PD

It is mainly of academic interest and is seldom required clinically. It is however useful in detecting female carriers and deficient patients during or soon after an episode of acute haemolysis. Kits are available from various manufacturers. Instructions given with the kit must be followed.

PYRUVATE KINASE (PK) DEFICIENCY

This is an enzyme of the Embden-Meyerhof pathway and its deficiency is the second most common after G-6-PD deficiency. There is no screening test available. The enzyme can be assayed in reference laboratories. Deficiency is suspected by:



- History - Autosomal recessive
- Chronic Non-spherocytic Haemolytic Anaemia
- Macrocytosis.
- Prickle cells in peripheral blood film.

PYRIMIDINE 5-NUCLEOTIDASE DEFICIENCY

This enzyme is required for degradation of RNA into soluble metabolites, which diffuse out of the cell. In its absence, RNA precipitates into small blue dot like deposits causing basophilic stippling. The enzyme is also inhibited by lead thus causing basophilic stippling in lead poisoning. The Enzyme can only be assayed in specialised laboratories.

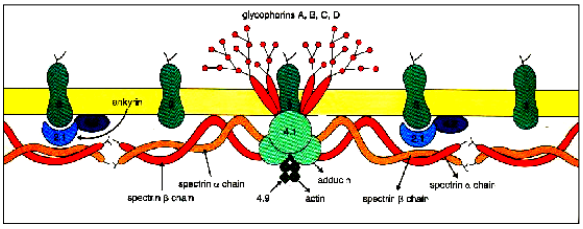


Figure 39.1: Structurae of red cell membrane

MEMBRANOPATHIES

These are the third common cause of congenital haemolytic anaemias. Normal shape of red blood cell depends upon structurally and functionally normal cell membrane and cytoskeleton. Cell membrane is a lipid bilayer in which certain proteins are inserted (Figure 39.1). These proteins are then anchored to cytoskeleton. Cytoskeleton is composed of various proteins. Abnormalities in these proteins result in various structural abnormalities of RBC rendering them susceptible to lysis in response to osmotic, temperature and metabolic changes. Important abnormalities are:

1. Hereditary spherocytosis
2. Hereditary elliptocytosis

3. Hereditary stomatocytosis

Common tests used for detection of these abnormalities are described below.

OSMOTIC FRAGILITY TEST

Principle

Normally salt and water, which enter the cell, are balanced by active pumping out of sodium along which water also diffuses out. In defective cells this balance is disturbed and water is retained by red blood cells. The cells, which are unable to accommodate excess of water e.g., spherocytes, swell and lyse in hypotonic solution earlier than the normal cells. In the test system small amounts of blood are mixed with large volume of buffered saline solutions of various concentrations. The fraction of RBC lysed in each concentration is estimated calorimetrically and plotted on a graph paper (Figure 39.2).

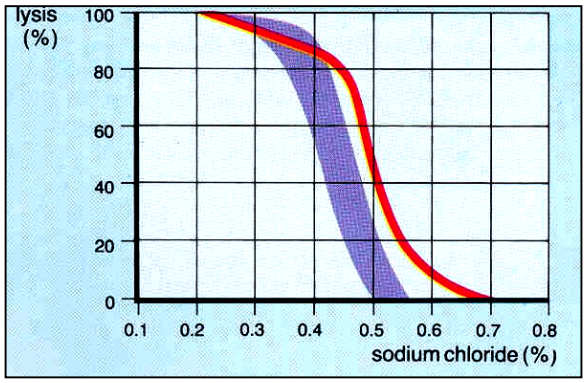


Figure 39.2: Osmotic fragility curve

Requirements

1. Stock solution of buffered sodium chloride 1.71 mol/L (osmotic equivalent of 100 g/L).
   a. Sodium chloride 90 g.
   b. Disodium hydrogen phosphate 13.65 g
   c. Sodium dihydrogen phosphate 2.34 g
   d. Distilled water to 1 L

   Store in refrigerator. Re-dissolve if any crystals are formed.
2. Working Solution (osmotic equivalent of 10g/L)
   a. Stock solution 10 ml
   b. Distilled water 90 ml
3. From this make dilutions equivalent to 9.0, 7.5, 6.5, 6.0, 5.5, 5.0, 4.5, 4.0, 3.5, 3.0, 2.0 and 1.0 g/L simply by taking working solution in volume in ml equal to the concentration required and making up the volume to 10 ml with distilled water.
4. Pipettes
5. Test tubes with stand
6. Spectrophotometer

Procedure

1. Collect venous blood in heparin from patient and from a healthy normal person. Defibrinated blood can also be used. The test should be carried out within two hours.
2. Take two sets (test and control) of 12 test tubes numbered 1 to 12. Add to each of tubes 1-11, 5 ml corresponding dilution. Add 5.0 ml of distilled water to tube 12.

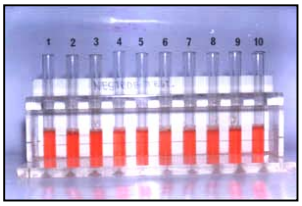


Figure 39.3: Osmotic fragility test

3. Add 50 µl of corresponding blood to each tube and mix immediately by inverting the tube several times without producing foam.
4. Allow to stand for 30 min at room temperature (15-25°C).
5. Mix again and centrifuge for 5 min at 1200-1500 g.
6. Using supernatant from tube 1 as blank read absorbance of all tubes at 450 nm in a spectrophotometer.
7. Assign 100% lysis value to tube 12.
8. Calculate % lyses for each tube by

$$\frac{\text{Abs of hypotonic tube}}{\text{Abs tube 12}} \times 100$$

9. Plot % lyses against NaCl concentration.
10. Calculate Median Corpuscular Fragility (MCF) (50% lysis).

INCUBATED OSMOTIC FRAGILITY

Blood samples are first incubated at 37°C for 24 hours and the test is performed as described above. An additional tube of 12 g/L should also be included in this test.

Reference range

Non-incubated MCF	4.0-4.45 g/L
Incubated MCF	4.65-5.9 g/L

AUTOHAEMOLYSIS TEST

Principle

The test provides information about metabolic competence of the red cells and helps in differentiating between enzyme and membrane defects. Blood is incubated both with and without glucose at 37°C for 48 hours and the amount of spontaneous haemolysis is measured calorimetrically.

Requirements

1. Glucose solution 100 g/L
2. Drabkin's solution
3. Screw capped bottles/tubes with stand
4. Pipettes

5. Spectrophotometer

Procedure

1. It is essential to use strict aseptic technique to avoid bacteria induced haemolysis.
2. Six ml of defibrinated blood sample is required for this test.
3. Put up 4 tubes, two marked plain (P) and two marked glucose (G).
4. Into each put 1 ml blood and save 1 ml, which is stored in refrigerator. Centrifuge 1 ml blood and save serum.
5. Into tube's marked G add 50 µl glucose solution.
6. Incubate all 4 tubes at 37°C for 48 hours mixing gently after 24 hours.
7. Pool 2 plain tubes separately and two glucose tubes separately.
8. Mix and determine PCV and Hb on portion of each.
9. Dilute a small amount from saved blood 1/100 in Drabkin's reagent.
10. Centrifuge remaining blood and separate supernatant.
11. Dilute supernatants from each of two tubes and supernatant from saved blood 1/10 in Drabkin's reagent. If there is marked haemolysis dilution can be increased up to 1/50.
12. Using pre-incubation serum from step 2 dilution as blank and blood tube from step 9 as standard, read all tubes at 625 nm in a spectrophotometer.

Calculation

$$\text{Lysis \%} = \frac{Rt}{Ro} \times \frac{Do}{Dt} \times (I - PCVt) \times 100$$

Where

Ro =Abs of dilute whole blood
Rt =Abs of dilute serum (after incubation)
Do =Dilution of whole blood
Dt =Dilution of serum
PCVt =Packed cell volume

Reference range

Without glucose 0.2-2.0 %
With glucose 0-0.9 %

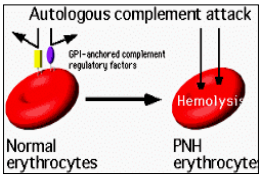
PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA (PNH)

It is an acquired clonal disorder in which RBCs are abnormally sensitive to normal constituents of serum.

Characteristically it presents as haemoglobinuria during sleep, haemosiderinuria and jaundice. The serum components, which are responsible for lysis, are those of normal complement. Screening tests for this condition are as under.

HEAT RESISTANCE TEST

Allow test and control sample of blood to clot at 37°C. Test is positive if free haemoglobin starts diffusing into serum soon after clot formation. Control serum remains clear.



SUCROSE LYSIS TEST

Principle

Red cells adsorb complement components at low ionic strength (isotonic sucrose solution) and lyse if PNH defect is present.

Requirements

1. Fresh solution of sucrose 92.4 g/L
2. Normal saline
3. Fresh serum collected from normal healthy person.
4. Normal AB or group compatible serum
5. Pipettes
6. Test tubes with stand
7. Centrifuge

Procedure

- Wash patient's RBC three times in normal saline and pack them by centrifugation as described earlier.
- Prepare 50% cell suspension.
- Put up two tubes one marked P (plain) and one marked S (sucrose).
- In both tubes place 0.05 ml normal serum.
- To tube P add 0.85 ml normal saline.
- To tube G add 0.85 ml sucrose solution.
- To both add 0.1 ml cell suspension.
- Incubate at 37°C for 30 min.
- Centrifuge the tubes.

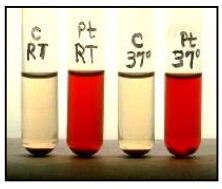
Result

Increased lysis in sucrose tube as compared to saline tube is a positive result.

HAM'S TEST (ACIDIFIED SERUM LYSIS TEST)

Principle

When PNH cells are exposed at 37°C to patient's own or normal serum at *p*H 6.5-7.0, they show abnormal lysis.



Requirements

- HCl 0.2 mol/L
- Normal saline
- Washed RBC from a normal healthy person

- Normal fresh AB or group compatible serum
- Drabkin's reagent
- Pipettes
- Test tubes with stand
- Centrifuge
- Water bath

Procedure

1. Separate normal and patient serum from freshly collected defibrinated blood.
2. Wash normal and patient red cells three times with normal saline and pack by centrifugation.
3. Prepare 50% suspension of both cells.
4. Inactivate portions of patient's and normal sera by heating in water bath at 56°C for 30 min.
5. Put up six tubes as shown in Table 39.1
6. Mix the contents carefully.
7. Incubate at 37°C for one hour. If the test is positive there is only trace haemolysis in tube 1 while tube 2 shows +++ haemolysis. All other tubes remain clear.

For quantitation prepare

- Blank tube containing 0.5 ml serum.
- Standard tube containing 0.05 ml cell suspension and 0.55 ml distilled water.
- Six tubes marked correspondingly containing 0.3 ml supernatant from each test tube.
- Add to each 5 ml Drabkin's reagent.
- Read all tubes against blank at 540 nm.
- Calculate % lyses for each tube by following formula:

$$\text{Lysis}\,\% = \frac{\text{Abs of Test tube}}{\text{Abs of Std tube}} \times 100$$

Result

Normal result shows not more than 2% haemolysis. PNH shows 10-50 % haemolysis.

Table 39.1: Procedure of acidified serum lysis (HAM's) test

Tube→	1	2	3	4	5	6
Fresh normal serum (ml)	0.5	0.5	-	0.5	0.5	-
Inactivated normal serum (ml)	-	-	0.5	-	-	0.5
HCl 0.2 mol (ml)	-	0.05	0.05	-	0.05	0.05
Patient RBC (ml)	0.05	0.05	0.05	-	-	-
Normal RBC (ml)	-	-	-	0.05	0.05	0.05

Note: If test is positive, repeat the whole procedure using patient's own serum to differentiate from HEMPAS. In later condition cells are not lysed in patient's own serum while in PNH test is positive even with patient's own serum. The sensitivity of test can be improved by adding 0.01 ml of magnesium chloride 250 mmol/L (23.7g/L) before incubation.

40. DIAGNOSTIC METHODS IN BLEEDING DISORDERS

Functional tests of coagulation are based on mimicking in vivo conditions in the laboratory. However the quantitative requirements of various coagulation factors to produce the end point in a particular test may not be the same as are in vivo. Therefore a gross discrepancy between the laboratory result and clinical condition may be seen. It is best exemplified by grossly prolonged PTTK in factor XII deficiency whereas the clinical manifestations of this deficiency are extremely mild. There are three types of assays available to quantitate coagulation factors. These are:

1. **Immunological assays**: These measure the coagulation protein, regardless of its functional capacity.
2. **Chromogenic peptide substrate assays**: In these assays activated factor is allowed to act on a synthetic peptide to which is attached a dye. The reaction releases the dye, which is then measured photometrically. However this activity is not the same as is the physiological activity of the activated factor. Therefore the results of these assays may also not reveal the physiological defect.
3. **Coagulation assays**: In these the coagulation factor is activated by means similar to those acting in vivo and is allowed to act on the natural substrate. Then the action is also compared with a control or standard. These are the best assays for clinical work.

GENERAL PRECAUTIONS

1. Only venous blood should be used.
2. Blood should be collected in liquid anticoagulant to allow quick and thorough mixing so that the process of coagulation does not get time to progress. The anticoagulant of choice is 31.3 g/L solution of trisodium citrate dihydrate or 38 g/L of trisodium pentahydrate.
3. The proportion of blood added to the anticoagulant must be exactly 9:1, otherwise results will not be comparable. If the PCV of the patient is less than 0.20 l/L or more than 0.60 l/L then the ratio of blood to anticoagulant will have to be changed. Following formulae may be used to determine the amount of anticoagulant to be added to a volume of blood or amount of blood to be added to a fixed volume of anticoagulant.

$$\text{Amount of anticoagulant} = 0.00185 \times \text{blood (ml)} \times (100 - \text{PCV})$$

$$\text{Amount of blood required} = \frac{60 \times 4.5}{100 - \text{PCV}}$$

4. Blood sample must be collected through a single clean venepuncture so that minimum tissue thromboplastin is introduced in the sample.
5. Samples must be collected in non-water wetable syringes into non-water wetable tubes. For this purpose disposable plastic syringes and tubes are economical.
6. The samples should be kept cold, preferably on ice, until processed.
7. Platelet poor plasma should be separated as soon as possible. This is done by centrifuging the sample at 2000 g for 15 min, preferably in a refrigerated centrifuge.
8. Tests should be completed within two hours of collection of sample. If samples are to be stored this should be done at -40°C.
9. Temperature of water bath must be accurately maintained at 37±0.5°C during test.
10. Before starting the tests stop watches and timers should be tested and wound, if not electronic.
11. Table lamp should be adjusted appropriately so that the clot detection is easy and quick. Opaqueness of the clot is inversely proportional to time, which it takes to form. Thus in tests with longer time the clot forms slowly. A uniform practice should be adopted to read the end point.
12. The trend for clotting to get prolonged with passage of time, due to deterioration of reagents, should be eliminated. If more than one sample is being tested in duplicate the arrangement should be something like A1 B1 B2 A2. The mean of two tests will take care of the difference in time.

PLAN OF INVESTIGATIONS

If a patient with a suspected coagulation disorder is to be investigated, the investigations should be pre-planned. The most important, in this regard, is the history and clinical findings in the patient. These help in deciding whether the patient has a vascular defect, platelet defect or a

defect in one or more of the coagulation factors. It also gives a clue as to whether the disorder is of hereditary or acquired nature and whether the inheritance is X-linked or autosomal (recessive or dominant). This appreciably narrows down the number of tests to be performed. The preliminary tests required are a platelet count (on page 254), bleeding time (on page 260), PT (on page 261), PTTK (on page 263) and thrombin time (on page 263). Further line of action is decided on the basis of results of these tests. Consult Table 40.1.

Table 40.1: Plan for investigations in a patient with bleeding disorder; N = Normal, = prolonged, = reduced

PLT COUNT	BT	PT	PTTK	TT	CAUSES	FURTHER TESTS
N	↑	N	N	N	Vascular abnormality, Plt function defect	Hess's test, Platelet function tests
N	↑	N	↑	N	von Willebrand disease	Platelet function tests, vWF assay`
N	N	N	N	N	Factor XIII deficiency, Severe trauma, Mild factor deficiency	FXIII assay FVIII & FIX assay
N	N	↑	N	N	Factor VII deficiency	FVII assay
N	N	N	↑	N	Intrinsic pathway factors deficiency	Mixing studies
N	N	↑	↑	N	Vit K deficiency, Oral anticoagulants, Liver disease, FII, FV, FVII and FX deficiency	History, LFT, Mixing studies
N	N	↑	↑	↑	Heparin, Hypofibrinogenemia Dysfibrinogenemia, Systemic hyperfibrinolysis	Thrombin time Mixing studies, Reptilase time
	?	↑	↑	N	Massive transfusion, Chronic liver disease	History, LFT
↓	↑	↑	↑	↑	DIC	FDP, D-dimers

Table 40.2: Plasma preparations required for mixing studies

PLASMA PREPARATION	DEFICIENT FACTORS
Fresh normal plasma	Nil
Plasma from patients on oral anticoagulants for 48-72 hrs	Factor VII
Plasma from patient on oral anticoagulants for a week or more	Factors II, VII, IX, X
Aged plasma	Factor V, VIIIC
Adsorbed plasma	Factor II, VII, IX, X
Serum	Factors I, V, VIIIC

Table 40.3: Correction of prothrombin time

Factor deficiency/ abnormality	Prothrombin time corrected by mixing with			
	Normal plasma	Adsorbed plasma	Aged serum	Coumarin plasma
Factor I	Yes	Yes	Yes	Yes
Factor II	Yes	Partial	Yes	Yes
Factor V	Yes	Yes	No	Yes
Factor VII	Yes	No	Yes	No
Factor X	Yes	No	Yes	Yes
Anticoagulants	No	No	No	No

Table 40.4: Correction of PTTK

Factor deficiency/ abnormality	PTTK corrected by mixing with		
	Normal plasma	Adsorbed plasma	Aged serum
Factor VIIIC	Yes	Yes	No
Factor IX	Yes	No	Yes
Factor XI	Yes	Yes	Yes

MIXING STUDIES

These experiments are carried out on mixtures of test plasma with either normal plasma or plasma of known factor(s) deficiency. The purpose of these tests is to determine the cause of prolongation of either PT or PTTK or sometimes of thrombin time. Factor deficient plasmas are commercially available but are very expensive. Plasmas with known factor deficiencies can be prepared in the laboratory and can then be used for mixing experiments. These are shown in Table 40.3 and Table 40.4. Once prepared, these plasmas can be stored in small aliquots at -20°C for future use.

PREPARATION OF ADSORBED PLASMA

Adsorbed plasma with same factors deficiency can be prepared by adsorption with **barium sulphate**. It is easy to prepare. The procedure is as follows:

1. To one ml normal plasma add 100 mg of barium sulphate.
2. Place the tube at 37°C and continue stirring for 3 min with a glass rod.
3. Centrifuge at 1200-1500 g for 10 min and collect supernatant.
4. Test prothrombin time, it should be more than 60 seconds. Otherwise carry out adsorption again.

PREPARATION OF AGED PLASMA

1. Collect blood in oxalate.
2. Centrifuge and separate platelet poor plasma.
3. Incubate at 37°C for 48 hours.
4. The prothrombin time of the aged plasma at the end of this period should be more than 90 seconds.
5. Plasma is then dispensed in plastic containers and stored at -35°C or lower.

Test Procedure

For mixing experiments same test is used which was abnormal. If prothrombin time was prolonged then it is repeated after mixing with appropriate reagents. If PTTK was prolonged then it is repeated after mixing with appropriate reagents. Correction of time is noted. To perform the test, one volume of test plasma is mixed with one volume of reagent plasma or serum. Details of test are same as described earlier (see PT on page 261 and PTTK on page 263).

Significance

See Table 40.3 and Table 40.4.

FACTOR ASSAYS

Precise activity of coagulation factors is assayed to:

1. Diagnose a bleeding disorder.
2. To assess the severity of disorder
3. To detect carriers
4. To monitor replacement therapy

Basic test used for assay depends upon the deficiency detected or suspected in screening tests described previously. Prothrombin time is used to assay factors II, V, VII and X. PTTK is used to assay factors VIII, IX, XI and XII. Factor deficient plasmas are required for assay. These are used as substrate. Serial dilutions of test and normal plasma are mixed with the substrate plasma and on each dilution corresponding clotting time is tested (i.e. prothrombin time or PTTK). Time obtained is plotted against dilution or percentage on a suitable graph paper and activity of factor in test plasma is estimated.

FIBRINOGEN ASSAY

Fibrinogen deficiency is indicated by prolonged thrombin time along with other abnormalities. It can be assayed by determining clotting time after addition of thrombin and comparing it with time obtained on known dilutions of fibrinogen. Reagents are available commercially in Kit form. The instructions and procedure supplied with the kit should be strictly followed.

FACTOR XIII DEFICIENCY

UREA SOLUBILITY TEST

Principle

Factor XIII is activated during clotting. Thrombin and calcium ions are necessary for its activation. Activated factor XIII stabilises the fibrin clot, which is not soluble in 5 mol/L urea solution for at least 1 hour, whereas clots formed in the absence of factor XIII dissolve rapidly.

Reagents

1. Patient's citrated plasma
2. Normal citrated plasma
3. Urea 5 mol/L (300 g/L)
4. Positive control prepared by mixing 0.2 ml EDTA plasma with 0.2 ml thrombin (20 NIH u/ml).

Procedure

1. Place 0.2 ml test plasma in a 75X12 mm glass tube.
2. Place 0.2 ml control plasma in another tube.
3. To each add 0.2 ml 10 NIH u/ml thrombin solution and incubate at 37°C for 20 min.
4. Treat positive control in similar way.
5. Add 3 ml urea solution to each tube and shake.
6. Leave overnight at room temperature undisturbed at 37°C.
7. Inspect next morning. Positive result is a clot, which dissolves in urea solution.
8. EDTA plasma can be used as a negative control.

MEASUREMENT OF FDP

Principle

The latex particles are coated with antibodies to FDP fragments D&E. If FDPs are present in the serum, they will agglutinate the latex particles.

Serial dilutions of the serum are used and agglutination with the highest dilution of serum is noted. This gives a semi quantitative estimation of the FDPs in the blood. The test is available in kit form commercially. Procedure is given with the kit. It is important that samples should be collected in an agent, which stops fibrin breakdown otherwise results will be falsely high. One such agent is ε-aminocaproic acid and tubes containing this reagent, for collection of specimens, are provided with kits.

PLATELET AGGREGATION TESTS

Platelet aggregation tests are indicated in cases of overt bleeding manifestations in which bleeding time is prolonged in the absence of significant thrombocytopenia and there are no abnormalities of coagulation pathway (except in von Willebrand Disease). A quantitative method has been devised to follow platelet aggregation by means of changes in light transmission of a sample of platelet rich plasma (PRP). A known quantity of an aggregating agent is added to citrated PRP, which is contained in a cuvette in a light recording machine under conditions of constant temperature and with continuous agitation. The changes in absorbance resulting from aggregation are measured directly or graphically. The result is dependent on the platelet count. This technique is not suitable with lipaemic samples. It is essential to obtain PRP from citrated venous blood collected into plastic tubes, and the tubes then capped to prevent loss of CO_2 from the blood to avoid change in pH. All handling of blood must be at room temp, as prior cooling inhibits the platelet aggregating response. In screening studies, PRP is generally challenged with a number of different aggregating agents, i.e., ADP, collagen, thrombin, adrenaline, and ristocetin.

Interpretation
See Table 40.5

Table 40.5: Interpretation of platelet aggregation studies. N = Normal aggregation, Abn =Impaired aggregation.

Disorder	ADP	Collagen	Adrenaline	Ristocetin	Arachidonic acid
Glanzmann's Thrombasthenia	Abn	Abn	Abn	N	Abn
Bernard Soulier syndrome	N	N	N	Abn	N
von Willebrand disease	N	N	N	Abn	N
Storage pool disease	N	Abn	Abn	N	N
Aspirin defect	N	Abn	Abn	N	Abn
Ehlers-Danlos Syndrome	N	Abn	N	N	N

THROMBOPHILIA

Thrombophilia are a group of conditions associated with an increased risk of thrombosis. These can be hereditary or acquired. Common causes of hereditary thrombophilia include Factor V Leiden, Protein C deficiency, protein S deficiency and Antithrombin III deficiency. Commonest acquired cause is Lupus anticoagulant.

LUPUS ANTICOAGULANT SCREEN

The lupus anticoagulant is most commonly an IgG immunoglobulin. It is an immediate acting coagulant inhibitor, which is characterised by a prolonged activated partial thromboplastin time (APTT). In mixing tests the APTT is not corrected by normal plasma. Although, APTT is prolonged, but it is rarely associated with bleeding problems. It is usually associated with thrombosis.

Principle
When APTT is performed in the absence of platelet substitute, it is particularly sensitive to lupus anticoagulant.

Requirements
1. Kaolin 20 mg/ml
2. Calcium chloride 0.025 mol/L
3. Platelet poor patient's plasma (depleted of platelets by second centrifugation, platelet count <$10x10^9$/L)
4. Normal platelet poor plasma
5. Plastic test tubes with stand
6. Glass test tubes
7. Water bath
8. Table lamp
9. Automated micropipettes and tips

Procedure
1. Blood sample collected and plasma separated as for clotting tests.
2. Arrange 6 plastic tubes in stand and prepare mixtures of normal plasma and patient plasma.
3. Pipette 0.2 ml of each mixture into a glass tube previously placed in water bath at 37°C.

Tube	1	2	3	4	5	6
Normal plasma ml	1	0.9	0.8	0.5	0.2	0
Test plasma ml	0	0.1	0.2	0.5	0.8	1

4. Add 0.1 ml Kaolin and incubate for 3 min.
5. Add 0.2 ml $CaCl_2$ and start stopwatch. Record the clotting time.
6. Plot clotting time (in seconds) against dilution NP/TP.

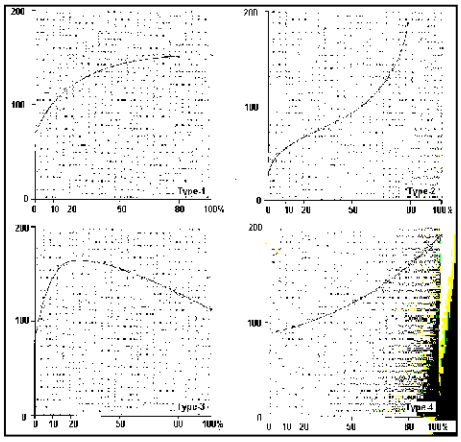


Figure 40.1: Lupus anticoagulant-Types of curves

Interpretation
1. Pattern-1: Curve convex near y axis-Classical lupus anticoagulant
2. Pattern-2: Sigmoid curve-coexistent factor deficiency and lupus anticoagulant
3. Pattern-3: Curve with peak near y axis-coexisting deficiency of lupus anticoagulant and inhibitory co-factor
4. Pattern-4: Rather straight line-No lupus anticoagulant

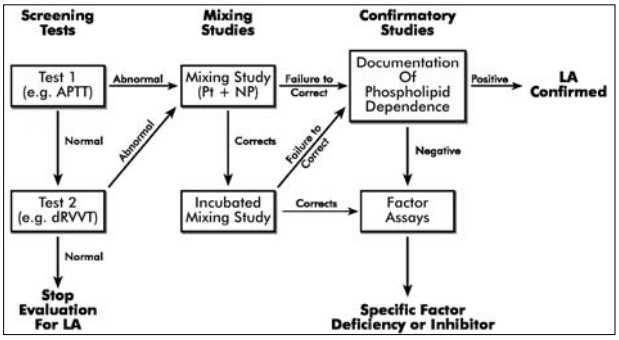
PLATELET NEUTRALISATION TEST

Platelets adsorb lupus anticoagulant. Therefore when platelets are used instead of phospholipid in the test system, the effect of lupus anticoagulant is neutralised. To utilise this property of platelets they must be washed to remove contaminating plasma proteins and antibodies to expose their collagen factor binding site.

DILUTE RUSSELL VIPER VENOM (DRVVT) TIME

Russell viper venom (RVV) activates factor X in the presence of phospholipids and calcium ions. The lupus anticoagulant prolongs the clotting time by binding to phospholipid and thus preventing the action of RVV. Whereas, in case

of factor deficiency the time is not prolonged. Since RVV activates factor X directly, defects of the contact system and factor VIII, IX or XI deficiencies will not influence the test.



ANTITHROMBIN

Antithrombin (AT), previously called antithrombin-III is the major physiological inhibitor of thrombosis and factors IXa, Xa, XIa and XIIa. AT deficiency is not uncommon and may be hereditary or acquired. In the presence of heparin, AT reacts rapidly to inactivate thrombin by forming a 1:1 complex. When serum is incubated with excess of thrombin, the residual amount of thrombin left at the end of incubation is proportional to AT activity. Normal levels of AT are usually in the range of 80-120 U/dl. Individual with congenital AT deficiency have a level around 50 U/dl. Newborns have a lower AT concentration than adults. A low level of AT may be acquired during active thrombosis, liver diseases or heparin therapy.

PROTEIN C AND S

Protein C is a vitamin K dependant protein. Protein C, in its native form, is inactive. It is activated by thrombin and thrombomodulin. It regulates blood coagulation by inhibiting factors Va and VIIIa. Protein C cleaves activated V and VIII. Protein C may be measured in three ways.

1. Clotting assay-generally measures function.
2. Antigenic assay-this measures total protein
3. Chromogenic assay-this measures binding site.

Protein C deficiency may be acquired as a result of liver disease, warfarin treatment and DIC or it may be hereditary. Protein S is also a vitamin K dependant protein and acts as a cofactor of activated protein C. Its deficiency may also be acquired or hereditary as of protein C. It is measured by the same methods.

ACTIVATED PROTEIN C RESISTANCE (APCR)

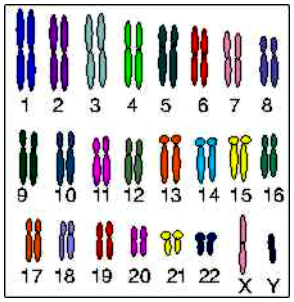
Activated factor V is a stimulus for thrombin generation. Activated factor V, produced during the course of coagulation process, is inactivated by activated protein C (APC). When it cannot be inactivated, it is called APC resistance. The stimulus for generation of thrombin continues resulting in thrombosis. The most common cause of APC resistance is an abnormal factor V protein called factor V Leiden. More than 90% cases result from a mutation (Arg506Glu). This mutation destroys a cleavage site for APC, hence greatly slowing the inactivation of factor Va. It is the most common cause of hereditary thrombophilia in white population. Its prevalence in Pakistan is low (~1%).

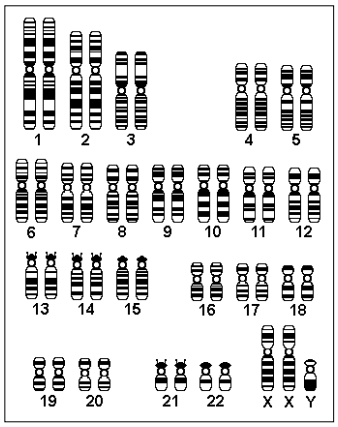
41. CLINICAL GENETICS

Pathology has traditionally been a descriptive science. In describing particular features of a morbid process pathologists have confronted only the consequences of biological processes and not the causative forces behind them. Our abilities to observe were greatly enhanced by light microscope and then the electron microscope. But at the sub-microscopic level explanation clearly lies with elements even smaller than the cellular components. The centre point of all cellular activities at the sub-microscopic level is DNA. It carries within its structure the hereditary information that determines the structure of proteins, which are the prime molecules of life. The past couple of decades have seen an extraordinary progress in understanding the structure and function of human genome. New techniques are now available for the study of normal as well as abnormal genes. This has opened new avenues for looking at things that are far beyond the reach of conventional diagnostic tools. For practical purposes Genetics can be subdivided into **cytogenetics** and **molecular genetics**. Cytogenetics deals with the study of whole chromosomes whereas molecular genetics involves the study of genes at the molecular level.

CYTOGENETICS

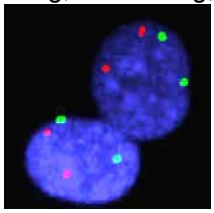
Chromosomes are thread like structures that lie coiled up in the nucleus of a non-dividing cell. At the time of cell division (metaphase stage) the nuclear material can be seen as individual chromosomes. The process of cell division, if arrested at this stage, provides an excellent opportunity to study the chromosomes. A normal human cell contains 46 chromosomes including 22 pairs of autosomes and one pair of sex chromosomes (XX in a female and XY in a male). Each chromosome consists of a pair of thread like structures united together at a constriction called centromere. Depending on the size of the chromosome and the position of the centromere the chromosomes can be divided in to seven groups (A-G). Karyotype refers to the number, size and shape of the total chromosomal content of an individual. A normal male karyotype is written as 46, XY and that of a female as 46, XX. Karyogram is a term that is used for the photograph of an individual's chromosomes arranged in a standard manner.





Method of chromosome analysis

The first step in the study of chromosomes involves culture of the cells. Most commonly the peripheral blood lymphocytes are used. However, the cells in solid tissues can also be studied. The lymphocytes in the presence of phytohaemagglutinin (PHA) are cultured in a suitable medium like RPMI 1640. After 72 hours the cell division is arrested at metaphase by colchicin. These cells are first suspended in a hypotonic KCl solution that causes them to swell and then they are fixed in acetic acid. A few drops of the fixed cell suspension are dropped on a glass slide that spreads the chromosomes. The chromosomes are visualised after Giemsa staining. In order to identify individual chromosomes a special procedure of banding is used in which the unstained chromosome slides are treated with trypsin. Subsequent Giemsa staining imparts each chromosome a unique banded appearance that can be seen under a light microscope. This type of banding is also called G-banding. Many different types of banding techniques like C-banding, Q-banding, and R-banding etc. are also used for identification of chromosomes in special circumstances. Recently it has also become possible to visualise individual chromosomes by using the technique of Fluorescent In Situ Hybridisation (FISH).



Common indications for cytogenetics

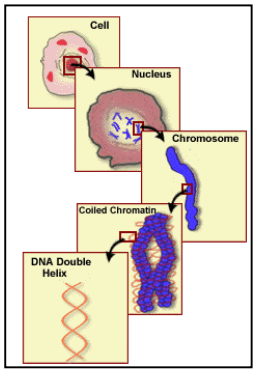
The abnormalities of chromosome number (aneuploidy) involve either loss or gain of one or more chromosomes. The structural abnormalities of chromosomes involve translocation of material from one chromosome to another, and deletion or inversion of material from individual chromosomes. The list of chromosomal disorders is very long whose description is beyond the scope of this discussion. The common indications where cytogenetics may be required are either constitutional disorders or malignancies and other acquired disorders. In both the categories the abnormality can be of either chromosome number or structure. Table 41.1 gives a list of the common disorders and the usual chromosomal abnormalities found in them.

Table 41.1: Common cytogenetic disorders and the abnormalities

Disorder:	Chromosomal abnormality:
Constitutional disorders	
Down's syndrome	Trisomy 21 (47, XX or XY +21)
Patau's syndrome	Trisomy 13 (47, XX or XY +13)
Edward's syndrome	Trisomy 18 (47, XX or XY +18)
Klinefelter's syndrome	47, XXY
Turner's syndrome	45, X
Fragile X syndrome	Fragile sites on X chromosome
Malignancies	
Acute lymphoblastic leukaemia	Hyperploidy, t(9;22) etc.
Acute myeloid leukaemia	t(15;17); t(8;21) etc.
Chronic myeloid leukaemia	t(9;22)
Non Hodgkin's lymphoma	t(14;18) etc.
Burkitt's lymphoma	t(2;8), t(8;14), t(8;22)

How to refer a patient for cytogenetics

The patients who require cytogenetic testing may be referred to the Department of Genetics AFIP. A brief history of the patient is recorded and 5 ml peripheral blood is collected in a sterile tube with Na-heparin (lithium free) as anticoagulant. In selected patients, particularly those with haematological malignancies, cytogenetics is done on bone marrow aspirates. Cytogenetics can also be done on Chorionic Villous Samples (CVS) and other solid tissues. The usual reporting time for cytogenetics is one month.



How to despatch sample for cytogenetics

The samples from patients who are unable to report in person to AFIP can be sent through courier. However, in all such cases it should be ensured that 5 ml blood from each person is collected in a sterile tube with heparin as anticoagulant. The samples must be accompanied by adequate medical summary of the case. It is important to ensure that all such samples should reach AFIP within 24 hours of collection. The samples can be safely transported at temperatures between 20-30°C.

MOLECULAR GENETICS

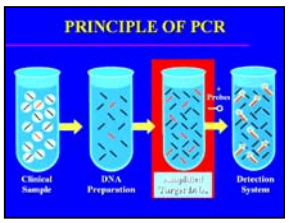
This deals with the genetic analysis at the subcellular level. The genetic material of a cell consists of Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA). Most of the cellular DNA is present in the nucleus with some traces in the mitochondria. RNA on the other hand is present in the nucleus as well as the cytoplasm.

DNA extraction

The first step in molecular genetics is the extraction of DNA from the test samples. DNA can be extracted from any dead or alive tissue containing nucleated cells. In routine practice 1-2 ml of peripheral blood collected in EDTA can yield up to 100μg of DNA. The red cells in the sample are lysed by a buffered solution containing 2% Triton-X 100. The white cells are sedimented by centrifugation at 3000 g for 5 min. The white cell pellet is lysed by overnight incubation at 37°C in 2% buffered solution of SDS and Proteinase-K. The proteins in the sample are precipitated by phenol chloroform extraction. DNA in the final solution is precipitated by 70% ethanol. The final DNA precipitate is re-dissolved in sterile distilled water. DNA can also be extracted from biological fluids containing nucleated cells, chorionic villus samples (CVS), archival bone marrow smears and paraffin embedded tissues, and other solid tissues. Several DNA extraction kit are now available.

DNA analysis

The DNA analysis mostly involves amplification by Polymerase Chain Reaction (PCR). The technique involves the use of a pair of 20-30 bp long pieces of DNA (primers) complementary to the DNA sequence of interest. The primers amplify the target sequence by means of repeated cycles of denaturation through heating of DNA, annealing of the primers to the single stranded DNA and extension of the primer DNA in the presence of four nucleotides (G, A, T, C), heat stable DNA polymerase (Taq polymerase) and a suitable reaction buffer. At the end of each cycle one



molecule of DNA would yield two molecules. If the cycles are repeated successively, 25-30 times for example, the target DNA can be amplified to over several million fold. This leaves sufficient amount of DNA that can be directly visualised after electrophoresis on agarose or polyacrylamide gels and staining with ethidium bromide or silver nitrate respectively. PCR is done on automated equipment (thermocycler see Figure 29.2) having a computer controlled heating block with capacity to hold 24-96 reaction tubes (see POLYMERASE CHAIN REACTION (PCR) on page 43 and on page 206).

Common uses of PCR in a diagnostic laboratory

Areas where PCR is used can be grouped as inherited disorders, malignant disorders, infectious disorders, forensic medicine, and tissue typing etc.

1. **Inherited disorders**: Most of the progress in this field is related to the disorders with a single gene defect. In this category of diseases, there is a clear Mendelian inheritance of a characteristic phenotype. All known autosomal dominant, autosomal recessive, and X-linked disorders belong to this category. Haemoglobin disorders including thalassaemia have been studied most extensively at the DNA level. Information is also rapidly emerging about mutations in disorders of the coagulation cascade, inborn errors of metabolism, endocrine disorders, lysosomal storage disorders, premature atherosclerosis, diabetes mellitus (insulin gene mutation), cystic fibrosis, muscular dystrophies, congenital renal diseases, and hereditary enzymopathies. Two main types of molecular lesions (mutations) have been observed to cause these disorders: gross abnormalities (deletions, insertions, or rearrangements) of genes; and a single nucleotide abnormality (point mutation) in critical region of the genes. The gross abnormality as well as the point mutation can be detected by various modifications of **Polymerase Chain Reaction** (PCR) where the abnormality is precisely known. Most commonly used PCR based technique is called **Amplification Refractory Mutation System** (ARMS). In disorders where the genetic lesion is not well characterised, an indirect approach of **Restriction Fragment Length Polymorphism** (RFLP) can be used. An exciting application is the diagnosis of inherited disorders during pregnancy (prenatal diagnosis). Prenatal diagnosis of a large number of inherited syndromes is now possible with the use of PCR. In practice, **Chorionic Villous Sampling** (CVS) is done under ultrasound guidance between 10-16 weeks of gestation. The sample is dissected under the microscope and clean foetal tissue is separated. DNA is extracted from the foetal tissue and the diagnosis of genetic abnormality is made. From the epidemiological point of view, prenatal diagnosis coupled with a therapeutic abortion in positive cases, has proved to be very effective in eliminating the genetic disorder from a community. With PCR it is also possible to diagnose an inherited disorder in an *in-vitro* fertilised embryo prior to its implantation (pre-implantation diagnosis).

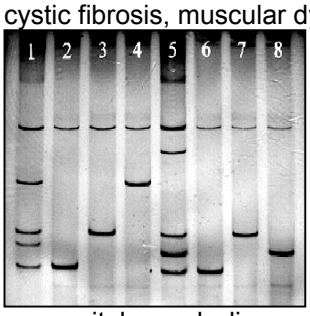
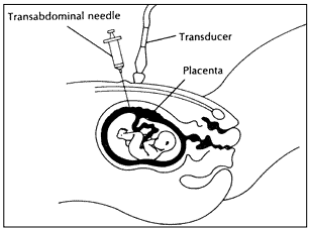


Figure 41.1: Silver stained polyacrylamide gel electrophoresis of ARMS PCR for -thalassaemia mutations. Lane-1 and 5 show allelic ladders. Other lanes show amplified PCR products for various mutations

2. **Neoplastic disorders**: The diagnosis of cancer by DNA analysis is based on the recognition that, at cellular level, neoplasia is almost certainly a genetic disorder. The genetic alterations responsible for the neoplastic proliferation of cells are usually acquired somatically only in the neoplastic tissues of the body. The genetics of cancer is intimately associated with two topics that have received considerable attention in the recent years: oncogenes and chromosomal rearrangements. More recently, the role of tumour suppressor genes in causation of cancer is also being recognised. The activation or aberrant expression of oncogenes lead in some way to excessive or uncontrolled cellular proliferation and seems to involve at least three different mechanisms: point mutations, gene amplification, and proximity to sites of chromosomal rearrangements. Each mechanism of oncogene activation carries a potential for diagnosis by DNA analysis.



Apart from oncogene analysis, the malignancies of lymphoid tissue can be diagnosed by demonstrating clonal rearrangements of Immunoglobulin genes or T-cell receptor genes. The approach is also useful for assigning the lineage commitment to lymphoid malignancies. In addition, an abnormal clone of lymphoid cells can be detected at a very early stage, or can be differentiated from a benign polyclonal lymphocytic proliferation. PCR can be used to detect the minimal residual disease in patients undergoing treatment for the malignant disorder. PCR can also be used to demonstrate the association of some malignancies and viruses e.g., human papillomavirus and the cervical cancer and HTLV-1 infection and leukaemia.

3. **Infectious disorders**: PCR is also becoming popular in diagnosis of infectious disorders. DNA based methods are very sensitive for the detection of pathogens. PCR is particularly very useful in tuberculosis where culture takes long time or leprosy where culture may not be possible. PCR is also being used for many fungal, parasitic and viral infections like hepatitis B and C, EBV and HIV etc. PCR based detection of viral genomes is an extremely sensitive and specific method. Gnomes as small as a single target molecule of DNA or RNA can be detected in a clinical sample. An interesting application in viral diseases is *in-situ* PCR. The virus particles, for example hepatitis B virus in the liver cells, CMV in lung, and EBV in association with lymphoma can be demonstrated in a tissue specimen. DNA techniques are also very useful for plasmid DNA analysis of various organisms that can be extremely useful in epidemiological survey of the infection.
4. **Miscellaneous applications**: The DNA amplification property of PCR has tremendous potential for applications in forensic pathology. It is based on the fact that the chance of DNA being similar from two different individuals is one in several million. Other useful applications include HLA typing for organ transplantation and identification of autoimmune linked HLA alleles.

42. TRANSFUSION MEDICINE

Transfusion medicine integrates the field of blood banking and clinical medicine in an effort to serve the ailing humanity with the best possible outcome. Assurance of safety in transfusion medicine depends upon the understanding of the subject and the application of knowledge in different clinical situations. One must be fully aware of the meaning of safe blood, which includes the knowledge of transmission of viral diseases like hepatitis and AIDS. The field of transfusion medicine has acquired its present status primarily due to a better understanding of immunology in general and immunohaematology in particular.

ANTIGENS

Antigen is a substance, which when introduced into an immunocompetent host, causes production of antibodies with which it reacts specifically. Considerable structural diversity exists among antigens (see also IMMUNOLOGY, on page 215). Blood group antigens are chemical structures embedded in or protruding from red blood cell, white blood cell and platelet membranes. The three most common forms of blood group antigens are glycoproteins (HLA system), glycolipids (ABO, Lewis, Ii, and P blood group systems) and proteins (Rh, M, and N blood group systems). Broadly speaking, antigens may be classified into two major types, exogenous and endogenous. In blood transfusion services, we are concerned primarily with antigens defined as allogenic (from another human) and autologous (self). These antigens are important in pregnancy, transfusion and transplantation.

IMMUNE RESPONSE

When a foreign antigen is introduced into the body for the first time, a primary antibody response characterised by a slow production of IgM antibodies occurs. When the same antigen is introduced for the second time, a secondary immune response occurs with the production of larger amount of antibodies, mainly of IgG type. This is called humoral immunity. For further details see section on IMMUNOLOGY on page 215.

ANTIBODIES

Antibodies are immunoglobulins produced by B-lymphocytes and plasma cells in response to antigenic stimuli. Upon exposure to appropriate antigens, plasma cells proliferate and synthesise immunoglobulins capable of specifically combining with the original antigen, a functional characteristic referred to as antibody specificity. In humans antibody is associated with five major classes of proteins, known as the immunoglobulins. These can be differentiated from one another on the basis of size, biological function, biochemical properties, and serological activity. Antibodies produced as a result of an antigenic stimulus are known as immune, acquired or warm antibodies. These are usually IgG e.g., Rh, Kell, Kidd, Duffy etc. Those antibodies that appear without any apparent antigenic stimulation like transfusion, pregnancy or vaccination are known as natural or cold antibodies. The latter are mostly IgM antibodies and are commonly found in ABO, M, N, Lewis, P and Ii systems of blood groups. Antibodies involved in blood group system are IgG, IgM and occasionally IgA. See Table 42.1 for properties of immunoglobulins and section on ACQUIRED IMMUNITY on page 215.

Table 42.1: Immunoglobulins in transfusion medicine.

PROPERTIES	IgG	IgM	IgA
Structure	Monomer	Pentamer	Monomer
Molecular weight	150,000	900,000	160,000
Carbohydrate percentage	3	12	8
Serum concentration (mg/dl)	150,00	200	350
Serum half-life(days)	23	5	6
Present in secretions	No	No	Yes
Antibody activity	Yes	Yes	Yes
Antigen binding sites per molecule	2	5-10	2
Complement fixation	Occasional	Yes	No
Cross placenta	Yes	No	No
Serological behaviour	Non Agglutinating	Agglutinating	Non Agglutinating

ANTIGEN ANTIBODY REACTIONS

A wide variety of antigen-antibody reactions are known but only those, which are important from transfusion point of view, are described.

Agglutination

Agglutination is the formation of aggregates of

particles, such as red blood cells that bear antigenic determinants on their surface, which combine with antibodies present in the test serum. The mechanism of agglutination is the formation of antibody bridges that connect the antigenic determinants of adjacent cells. Agglutination can be observed through both direct (ABO grouping) and indirect techniques (antiglobulin procedures). Agglutination occurs in two stages:

1. The antibody attaches itself specifically to the polysaccharide/lipid/protein complexes that form the antigen sites on the red cell. This process is known as sensitisation, which requires proper temperature, *p*H and ionic strength of the medium and antigen-antibody ratio.
2. The second phase is the physical process of agglutination, in which cells come together to form clumps. This depends upon the type of antibody involved, antigen sites available and zeta potential of the medium in which the cells are suspended.

Zeta potential: Red cells, when in suspension, carry a negative charge on their surface in the form of sialic acid residues. These charges serve to repel the adjacent cells to avoid sledging and achieve satisfactory oxygen carriage. When the cells are suspended in an electrolyte solution, electropositive charges are attracted towards the cells thus carrying a double ionic cloud, which moves alongwith each cell. The farther end of this edge is known as the surface of shear or the slipping plane. This determines the effective charge of the red blood cells and is designated as zeta potential. In order to bring an antibody close to the surface of the cell, this potential has to be reduced. Even those antibodies that cannot exhibit agglutination normally can do so if this potential is reduced. This can be brought about by the following procedures:

1. **Proteolytic Enzymes**: The enzymes used to enhance antigen antibody reactions include papain, ficin and bromelin. They also remove structures on the red cells membrane, so as to facilitate the interaction of antibody with the corresponding antigen. The red cell antigens that are enhanced by enzymes include Rh, Kell, Kidd & Lewis blood groups. Certain red cell antigens, including Duffy (Fy) and M, N, S and s, are destroyed by enzymes.
2. **Albumin**: It is prepared from bovine source and is commercially available as 22% preparation. Over time the use of albumin has been abandoned by many blood banks, due to availability of better potentiators like LISS and polyethylene glycol.
3. **LISS**: Low ionic strength saline is widely used in blood banks as an enhancing medium. The incubation time is shortened to 10 min and most antibodies are well detected by this technique. There are reports of diminution of reactivity of anti-K in low ionic strength medium.
4. **PEG (polyethylene glycol)**: It potentiates agglutination by taking out water of hydration, thus binding the cells together and enhancing 2nd stage of agglutination.

Haemolysis

This is an important antigen-antibody reaction in which final result is lysis of cells. Red colour of free haemoglobin released during immune destruction of cells is an important end point of antigen antibody reaction. Haemolysis represents destruction of the red blood cell membrane through the action of complement proteins that are activated by attachment of specific antibody to a surface antigen. Haemolysis is a positive result indicating the presence of a complement-activating antibody. Antibody-mediated haemolysis does not occur in the absence of complement or in plasma when a calcium-chelating agent is present. Haemolytic reaction occurs in two stages:

1. The antibody combines with the antigen, thus sensitising the red cell.
2. Sensitised red cells lyse with the help of activated complement components.

Table 42.2. Reading and interpreting agglutination reactions and haemolysis

Symbol	Agglutination score	Description
4+ or C (complete)	12	Cell button remains in one clump, macroscopically visible
3+	10	Cell button dislodges into several large clumps, macroscopically visible
2+	8	Cell button dislodges into many small clumps, macroscopically visible
1+	5	Cell button dislodges into finely granular clumps, macroscopically just visible
(+) or w (weak)	3	Cell button dislodges in fine granules, only visible microscopically
-	0	Negative result-all cells free and evenly distributed

REQUIREMENTS OF A STANDARD BLOOD BANK

AREA

- Work area required varies greatly with number of expected blood donations and workload. However it should be divided into following sections.

- Reception and donation section comprising of waiting area, donor's assessment room where donor is weighed and haemoglobin is tested, donation room and an area marked for the refreshment of donors.
- Screening section
- Blood banks for storage of blood
- Laboratory including x-match & Issue section
- Stores
- Offices
- Components section (if facilities available)

STAFF

- Pathologists (Haematologists) with experience in transfusion medicine
- Preferably a microbiologist for screening tests
- Laboratory technicians trained in transfusion medicine
- Phlebotomy nurses
- Auxiliary staff

EQUIPMENT

Following equipment is required for a routine blood bank. In case more specialised services are acquired then additional equipment may also be needed.

- Donation beds
- Height and weight scales
- Mixing and weighing equipment (haemoscale) for blood units during donation or at least hanging weighing scales.
- Sphygmomanometer
- Stethoscope
- Oxygen inhalation equipment
- Suction machine
- Air-ways for emergency use
- Normal Saline infusion with IV set (500/1000 ml).
- Crepe Bandage
- Blood bank refrigerators with continuous temperature recorder and alarm.
- Blood bag centrifuge, preferably refrigerated[1].
- Deep freezer (-30 to -80°C) for freezing plasma and cryoprecipitate.
- Platelet incubators[1]
- Laboratory incubator (37°C)
- Refrigerators
- Water baths with temperature control
- Laboratory centrifuge
- High speed centrifuge for 75x10 mm test tubes
- Glass test tubes 75x10 mm
- Metallic test tube racks with 12 holes in each row to hold 75x10 mm test tubes.
- Glass Pasteur pipettes
- Grouping tiles
- Glass slides
- Microscope
- Automatic ELISA equipment if screening workload is more than 50 samples per day.
- Tube Sealer
- Stationery and rubber stamps marked with labels of blood groups, components and names of tests
- Computers

REAGENTS

- Full range of blood grouping sera (anti-A, anti-B, anti-AB, anti-D)
- Bovine albumin 22% / LISS
- Polyspecific Coomb's reagent
- Three cell panel for antibody screening
- Eleven cell panel for antibody identification
- Phosphate buffered saline (can be made in the laboratory)
- Low ionic strength saline, LISS (can be made in the laboratory)
- Ether

PREPARATION OF BASIC REAGENTS

BUFFERED NORMAL SALINE

1. Phosphate buffer *p*H 7.0
   a. Solution A: $NaH_2PO_4.2H_2O$ 23.4 g/L
   b. Solution B: Na_2HPO_4 (anhydrous): 21.3 g/L
   c. Mix: 32 ml solution A & 68 ml solution B
2. Normal saline 9.0 g/L
3. Buffered saline: Mix equal volumes of solution 1 & 2

LOW IONIC STRENGTH SOLUTION

Low ionic strength solution or LISS reduces the zeta potential and thus enhances association of antibody with antigen. The major advantage is that the incubation period in indirect antiglobulin test can be reduced while maintaining or increasing the sensitivity of detection of majority of red cell antibodies. The red cells should be washed in saline and suspended in LISS. One volume of cell suspension and two volumes of serum should then be used for the test. Incubation period can be reduced to 10 min. its *p*H is 6.6-6.8, osmolality 270-285 and conductivity of 3.5-3.8 mS/cm.

[1] If facilities exist

PREPARATION OF LISS

1. Stock Solutions: Dissolve 42.9 g Na_2HPO_4: and 10.2 g KH_2PO_4: separately in 500 ml water.
2. Working Solution: Dissolve 1.75 g NaCl, 18.0 g Glycine[1] in water. Add 8.7 ml Na_2HPO_4, 11.3 ml KH_2PO_4 and make volume up to 1 litre with distilled water
3. Adjust *p*H to 6.7 with NaOH
4. Add 0.5 g of Sodium azide as preservative.
5. The LISS should have the following characteristics:
   a. pH: 6.6.-6.8
   b. Osmolality: 270-285 mmol
   c. Conductivity: 3.5-3.8 ms/cm at 23°C

BLOOD DONATION

RECEPTION OF DONORS

A blood donor is not an ordinary person, particularly in our community where baseless prejudice and fears against blood donation still persist. He deserves our special attention and care. It must be recognized that:

1. The environment of the donation centre must be clean, comfortable and quiet. It should be well lighted, well furnished, well ventilated and preferably air-conditioned.
2. Personnel on duty should exhibit an attitude of professional competence and good mannerism.
3. Every donor is a VIP and should be treated accordingly. Unnecessary and non-professional arguments should be avoided with the donor.
4. Introduce yourself by name to the donor, offer him/her a seat and then ask about the recipient for whom he/she wants to donate blood, or if he/she is a voluntary donor.
5. Explain the procedure to the donor, reassure him/her that the procedure is safe, and will entail only a single prick of needle.
6. Give him/her the chance to ask questions.
7. First time donors must be handled very carefully and reassured.

DONOR SELECTION / REGISTRATION

Considerable care must be exercised on selecting potential blood donors for the protection of both donor and the recipient. Most donors may be accepted on the basis of medical history, general appearance and haemoglobin estimation, although it is advisable to examine all vital signs.

GENERAL APPEARANCE: The donor should appear to be in good health. Poor physique, debilitation, under nutrition, plethora, jaundice, cyanosis, dyspnoea and mental instability should be noted. Clinical examination suggestive of intoxication either by alcohol or narcotic drugs should be a reason to exclude that donor. The skin at the venepuncture site should be free from lesions.

WEIGHT: The donor must weigh more than 50 kg to donate a full 450 ml donation of blood. Those weighing less but are otherwise healthy may donate 250 ml of blood for which either a reduced volume of anticoagulant or special donation bag is used.

AGE: First time donors should be 18-60 years old. Donors may continue to donate regularly till 65 years of age.

HAEMOGLOBIN: Haemoglobin concentration should be determined before every donation. Haemoglobin screening can be done by copper sulphate specific gravity method, Hemacue strips, spectrophotometric method or haematology analyser. The acceptable level is 13.0 g/dl for males and 12.0 g/dl for female donors.

MEDICAL HISTORY: A thorough medical history should be taken to ensure that the donor is free of all diseases. Special emphasis should be given to ask about the history of viral hepatitis within 1 year, venereal diseases, AIDS, cardiovascular diseases, hypertension, renal diseases, diabetes mellitus, tuberculosis, bleeding disorders, central nervous system disorders, gastrointestinal disorders, respiratory diseases and malignancies. Donors having above-mentioned diseases are excluded from blood donation.

PRESENT CONDITIONS: Pregnancy, lactation, infections, and blood donation within less than 12 weeks are reasons for temporary rejection for blood donation.

DONOR REGISTRATION CARD: Blood donor registration card is filled in the presence of the donor. The details of physical examination and medical history are printed on the card. The questions must be asked in the language the donor would understand.

Copper sulphate method for Haemoglobin concentration screening

Aqueous copper sulphate, coloured blue, with a specific gravity of 1.053, equivalent to 12.5 g/dL haemoglobin is normally used to test female donors. Copper sulphate, coloured green with a specific gravity of 1.055 equivalent to 13.5 g/dL can be used to test male donors. The donor's

[1] Glycine prevents nonspecific uptake of complement on the red cell surface.

fingertip is cleaned with a swab of methylated spirit and punctured by a lancet. The first drop of blood is wiped off by a piece of sterile gauze. The second drop is allowed to reach as big a size as possible and allowed to drop by itself from a height of 10 mm into appropriate copper sulphate solution. The drop is observed for 15 seconds. If the drop of blood has a higher specific gravity than the copper sulphate solution, it will sink within 15 seconds. If not, then it either takes longer time to sink or remains suspended or even may rise to the top of the solution. The results are interpreted as “Pass” or “Fail” accordingly.

COLLECTION OF BLOOD

After medical history, physical examination and checking the haemoglobin level, the donor is guided to the blood donation room. The name and other particulars of the donor are counterchecked and following procedure is adopted:

1. Blood should be drawn from a suitable vein in the antecubital fossa in an area that is free of any skin lesions.
2. Clean it thoroughly with iodine and methylated spirit.
3. A sphygmomanometer cuff is wrapped around the upper arm.
4. Inspect the bag containing the anti coagulant (CPDA-1, shelf life 35 days). It should be clear and colourless.
5. Label the bag and two plain glass test tubes/screw capped bottles (to be used later as pilot tubes).
6. Now raise the pressure in the sphygmomanometer cuff to 50-80 mm of Hg. The veins will become prominent.
7. Perform phlebotomy. The blood will start flowing into the bag.
8. The attendant should observe and ensure that the blood is flowing at a steady speed and he/she should gently mix the blood in the bag or automatic mixing/weighing equipment should be used.
9. The attendant should also look for the condition of the donor. If he/she manifests signs of fainting, sweating or palpitation the process should be stopped at once.
10. When the required quantity of blood has been collected, which takes less than 10 min, the pressure in sphygmomanometer cuff is released. Two clamps are applied as close to the needle as possible. The tubing is then cut between the clamps with small scissors. Take a sample of blood in pilot tubes by releasing the clamp near the needle and then apply it again.
11. Sterile gauze is placed over the puncture site, needle is withdrawn and puncture site is sealed aseptically with adhesive dressing.
12. The arm and general well being of the donor should be checked.

STORAGE OF BLOOD

Blood must be stored in a blood bank that operates between 2-6°C, well-lighted, equipped with alarm and temperature recording devices. The red cell concentrates/whole blood can be stored for 35 days from the date of collection, in blood bags containing CPD-A1, as the solution. Citrate is a calcium-chelating agent, which prevents the blood from clotting. Dextrose is provided as a nutrient for red cells to support the generation of ATP by glycolysis, thus increasing red cell viability. The addition of adenine is also associated with improved synthesis of ATP in stored blood.

AFTER CARE OF THE DONOR

1. Make sure that bleeding has stopped from the phlebotomy site.
2. Let the donor remain lying on the couch for at least 10 min so that his/her circulation is readjusted.
3. The donor is provided with light refreshments, particularly tea/coffee and request to refrain from smoking for an hour or so.
4. Before the donor leaves blood donation centre it is ensured that he/she is perfectly all right and there is no bleeding from phlebotomy site.

SCREENING OF BLOOD

Once the blood has been donated, pilot tubes are sent to the screening department. Following tests should be performed routinely on donor's blood:

1. ABO and Rh typing.
2. Screening for haemolysins (Group O individuals).
3. Screening for antibodies other than the ABO group antibodies.
4. Hepatitis B and Hepatitis C screening (HBsAg, Anti-HCV antibody).
5. Screening test for AIDS (Anti-HIV antibody).
6. Screening test for Syphilis (VDRL).
7. Screening for malarial parasites in high-risk areas.

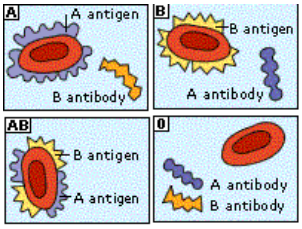
Any positive reaction observed in screening tests makes the blood unit unfit for transfusion and it should be discarded.

BLOOD GROUP SYSTEMS

There are many blood group systems including ABO, Rh, Kell, Kidd, Duffy, Lutheran, Lewis, MNS. However, in routine only ABO and Rh systems are important.

ABO AND Rh D GROUPING

It is the most important part of blood screening. It can be performed on the cells as well as on the serum. When it is performed on the cells it is called direct grouping (forward typing) in which unknown red cells (test cells) are tested against known antisera. When it is performed on the serum it is called indirect grouping (reverse typing) in which the unknown serum (test serum) is tested against known red cells. Ideally both should be performed on each specimen.



DIRECT GROUPING (FORWARD TYPING)

This can be performed either by tile method or by tube method.

Tile method

1. Allow the blood to clot. Clear supernatant serum should be aspirated carefully with the help of a Pasteur pipette into another clean tube.
2. Prepare 5% cell suspension from the cells by mixing one drop of packed cells with 19 drops of buffered normal saline.
3. Divide the tile with grease pencil into A, A1, B, AB and Rh D squares.
4. Place one drop of corresponding antiserum in each square.
5. Add a drop of test cell suspension into each of the squares containing antiserum.
6. Mix with glass rod, cleaning its tip thoroughly after mixing in each square.
7. Tilt the slide gently backward and forward at room temperature for a maximum of two min.
8. Read macroscopically for agglutination and record the result.
9. Rh D negative persons may be tested for D^u (variant).

Test tube method

1. Prepare 2-3% suspension of red cells in isotonic buffered saline.
2. Arrange test tubes in the rack, marked anti-A, anti-A1, anti-B, anti-AB and anti-Rh D.
3. Add one drop of corresponding antiserum to each of the test tubes.
4. Using a small pipette add one drop of 2-3% cell suspension to all the test tubes.
5. Mix well and centrifuge at 3400 RPM (900-1000g) for 30 seconds.
6. Try to re-suspend the cells by gentle agitation and read macroscopically for agglutination and/or haemolysis.
7. Confirm negative result by microscopy.
8. Rh D negative persons must be tested for D^u (variant).

Quality control: The antisera should be tested with known A, B and O red cells with each batch on a tile or in tubes to determine their effectiveness.

INDIRECT GROUPING (REVERSE TYPING):

This can also be performed by tile or tube method.

Tile method

1. Divide the tile with grease pencil into A, B, O and autocontrol squares.
2. Place one drop of patient's serum in all the squares.
3. Add one drop of corresponding 2-3% suspension of red cells to labelled squares.
4. Add one drop of 2-3% suspension of patient red cells to the square labelled autocontrol.
5. Mix well with glass rod, cleaning its tip after each application. Tilt the slide gently backward and forward at room temperature for a maximum of two min.
6. Read macroscopically for agglutination.

Test tube method

1. Place 2 drops of serum to be tested into test tubes labelled A, B, O and autocontrol.
2. Using Pasteur pipette add one drop of 2-3% suspension of corresponding cells into each tube.
3. Add one drop of test red cells (patient's red cells) to the test tube labelled "autocontrol".
4. Mix well and centrifuge at 3400 RPM (900-1000g) for 30 seconds.
5. Try to re-suspend the cells by gentle agitation and read macroscopically for agglutination or haemolysis.
6. Confirm negative result by microscopy

Quality control: The reagent red cells used for reverse grouping should be crosschecked against known anti sera with each batch. It is better to adopt a standard procedure for recording the results on a work sheet. A sample work sheet is shown in Table 42.3.

Table 42.3: Work sheet for blood grouping work

Donor/ Patient ID	Anti-A	Anti-A1	Anti-B	Anti-AB	Anti-D	A cells	B cells	Auto control	Result
1	+++	+++	-	+++	+++	-	+++	-	A, 'D' Pos
2	+++	-	-	++	+++	-	+++	-	A2 'D' Pos
3	-	-	+++	+++	-	+++	-	-	B, 'D' Pos
4	+++	++	+++	+++	++	-	-	-	AB, 'D' Pos
5	-	-	-	-	+	+++	++	-	O, 'D' Pos
6	-	-	-	-	-	+++	++	-	O, 'D' Neg

Table 42.4: Causes of discrepancies in ABO and Rh grouping

False positive result	False negative results
Rouleaux formation	Impotent sera
Auto/allo antibodies	Failure to add grouping sera
	Cell lysis in reverse grouping

Table 42.5: ABO Blood groups, subgroups, antigens and antibodies.

Blood group	subgroup	Antigens on cells	Antibodies in plasma
A	A_1	$A + A_1$	Anti B
	A_2	A	Anti A_1#
B	-	B	Anti A
AB	A_1B	$A + A_1 + B$	None
	A_2B	A + B	Anti A_1#
O	-	-	Anti A
	-	-	Anti B

D^u TESTING

1. Place one drop of anti-D serum in a test tube.
2. Add to it one drop of patient's cell suspension
3. Incubate the test tube at 37°C for 30-60 min.
4. Wash the cells five times with saline.
5. Add 2 drops of antiglobulin (Coomb's) serum mix and centrifuge at 3400 RPM for 10 seconds.
6. Read for agglutination. Agglutination in the test indicates D^u variant.
7. If there is no agglutination, add one drop of check cells to test tube. Centrifuge at 3400 RPM for 10 seconds and read for agglutination. If the check cells also show no agglutination antiglobulin (Coomb's) test is invalid and must be repeated.

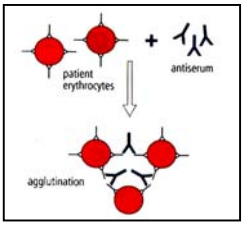
Quality control: Anti-D serum should be tested against known Rh-positive and Rh-negative red cells with each batch of tests.

ANTIGLOBULIN TEST (COOMBS TEST)

In some cases, a small antibody molecule such as IgG can sensitise red blood cells but cannot produce agglutination. The small size of the antibody molecules makes them unable to overcome the forces that cause red blood cells to repel one another and hence fail to form cross-linked bridges that connect cells. In 1945, Coomb et al described a test for detecting these non-agglutinating, coating (sensitising) antibodies. Later, the same test was used to demonstrate coating of red blood cells with complement components as well. This test is known as the antiglobulin test or Coomb's test. The antiglobulin test is performed in two ways, direct antiglobulin test (DAT) and indirect antiglobulin test (IAT).

DIRECT ANTIGLOBULIN TEST (DAT)

The direct antiglobulin test brings about agglutination of human red cells that have already been sensitised in vivo by antibodies or complement components. The Coomb's serum containing both anti human globulin and anti-complement antibodies can detect both of these sensitised cells by inducing visible agglutination.



Indications

It is indicated for determination of antibody-coated red cells in haemolytic disease of newborn, autoimmune haemolytic anaemia and following haemolytic transfusion reactions.

Procedure

1. Wash red cells of patient three times with normal saline.
2. Add volume (drop) of 3% washed red cell suspension in a test tube.
3. Add 2 drops of Coomb's reagent.
4. Mix and centrifuge for 15 seconds.
5. Re-mix cells gently and observe for agglutination.
6. Confirm microscopically, for presence of agglutinates or otherwise.

Interpretation

Agglutination indicates positive test that means that the red cells have been sensitised in vivo either with antibody alone or with components of complement. A valid negative test indicates lack of in vivo sensitisation or insufficient globulin or complement molecules on the red cell surface to allow detection.

INDIRECT ANTIGLOBULIN TEST

The indirect Coomb's test is used to demonstrate circulating antibodies in the serum, which do not agglutinate cells suspended in saline. This depends on the combination in vitro of an antibody with its specific antigen. In the indirect test normal O^+ group red cells are exposed to a serum suspected of containing an

In 2% of A_2 subjects and 25-30% A_2B subjects.

antibody and subsequently tested after washing to see whether they have been sensitised or otherwise. Two steps are required. First step involves incubation of the serum with known O group red cells to allow them to become coated with antibody if present in the serum. Second step involves testing for sensitised cells as in direct Coomb's test.

Indications

1. Compatibility testing (cross match).
2. Detection and identification of irregular antibodies.
3. Detection of antibodies e.g., Kell, Duffy and Kidd etc.
4. Investigation of Immune Haemolytic anaemias.

Procedure

1. Two volumes (drops) of serum are placed in a test tube.
2. One volume (drop) of 3% red cell suspension is added to it.
3. Mix thoroughly.
4. Incubate at 37°C for 50 min.
5. Wash these cells three times with normal saline.
6. After removal of saline of the last wash add 2 drops of Coomb's reagent.
7. Mix and centrifuge for 15 seconds.
8. Re-mix cells gently and observe for agglutination. Confirm microscopically.
9. If the test is negative add 1 drop of check cells to confirm validity of Coomb's serum.
10. Reduce incubation time to 10 min if equal volume of LISS is added to the patient's serum.

Interpretation

The presence of agglutination indicates the presence of antibodies in the test serum capable of reacting with the test cells. If known antiserum is used, the test will indicate presence of corresponding antigen.

Quality control: The antiglobulin serum should be checked against known sensitised cells. The sensitised red cells may be commercially purchased or prepared in the laboratory.

Preparation of check cells: take 1 ml serum from a D negative patient who has already been sensitised by exposure to D positive foetal cells during pregnancy/delivery. The titre of anti D antibodies should be at least 1/16. Mix this serum (containing anti D IgG) with I ml washed O positive cells and incubate at 37°C for 30 minutes. Wash the cells and make a 3% suspension with saline. These IgG coated check cell may be used to check validity of Coombs test.

SOURCES OF ERROR IN ANTIGLOBULIN TEST

False negative test

1. Test tubes or pipettes may be dirty.
2. Red cells may have been inadequately washed.
3. Proteins on fingertips may neutralise AHG and thus false negative result may be obtained.
4. Incubation time is too short or too long.
5. Incubation is at a temperature at which the antibody is not active.
6. There is a delay in reading the test or in performing the test thus allowing the antibody to be eluted off the red cells.
7. Test cells are stored improperly causing them to loose activity.
8. The antiglobulin serum is inactive because of improper storage or it is not added at all.
9. Change in the optimal ratio of antibody to antigen.
10. If plasma rather than serum is used.
11. Under-centrifugation.

False positive test

1. Presence of heavy metal ions and colloidal silica in saline solution can cause non-specific agglutination.
2. Bacterial contamination of test cells because of improper storage.
3. Refrigerated clotted blood results in a non-specific binding of C4, which can react with the anti-complement of the antiglobulin serum.
4. Over centrifugation will result in a false positive test.

COMPATIBILITY TEST (CROSS MATCH)

The purpose of the cross match test is to ensure serological compatibility between the recipient's serum and the donor red cells. This includes ABO compatibility and detection of red cell alloantibodies in the patient's serum. In many transfusion centres the cross match procedure has been replaced with 'type and screen' policy, according to which both the donor and the recipient are typed for ABO & Rh 'D' groups and screened for atypical antibodies. The blood is then released either by performing an **Immediate Spin** or **Computer Cross match**. However, in centres in which antibody screening is not done, the following tests should be included as part of compatibility testing:

1. ABO & Rh 'D' grouping of donor unit.
2. ABO & Rh 'D' grouping of Patient/ recipient.
3. Indirect antiglobulin test (IAT) using serum of patient and donor red cells. The

incubation time can be reduced to 10 min if LISS is used as potentiating agent (Table 42.6).
4. The cross-match should include testing at room temperature, LISS phase at 37°C, and indirect antiglobulin test at 37°C.
5. Release of blood after sticking the name of recipient on the donor blood bag and filling of appropriate issue form.

Rh D ANTIBODY TITRATION

Antibody titration is a semi-quantitative means of assessing the amount of antibody in the serum. This is usually done in Rh-incompatible mothers with a view to induce labour if the titre progressively rises. It should be done after detection of antibody by IAT and identification by cell panels.

Procedure
1. Set up 10 test tubes in a rack. Label them as 1/1, 1/2, 1/4,1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512 and 1/1024.
2. Add 2 drops of saline in each starting from second (1/2) tube.
3. Add 2 drops of patient's serum in the first and second tubes.
4. Mix well and transfer 2 drops from 2nd test tube to the third. Mix well and transfer 2 drops to the next tube and so on till last tube is reached. Discard 2 drops from the last tube.
5. Add one drop of 2-5% known O Rh D positive cell suspension in saline in each tube and centrifuge for 15 seconds at the rate of 3400 RPM. Look for agglutination.
6. Incubate the test tubes at 37°C for 50 min.
7. Enhancement media use is not recommended because it is difficult to maintain ratios.
8. Wash the cells three times with normal saline.
9. After the last wash add 2 drops of Coomb's serum in all the test tubes.
10. Centrifuge for 15 seconds at 3400 RPM and read results.

Interpretation
Highest dilution showing agglutination indicates titre of the antibody in the serum. While reporting the titre, the dilution prior to the highest one showing agglutination is reported.

Caution
Store the previous sample frozen in lab and retest it alongwith the next sample of same patient for antibody titration. Results should always be compared with those previously reported.

Table 42.6: Work sheet for recording compatibility test results.

Patient ID: Patient Blood Group:

Donor ID/ Bag number	Donor blood group	Saline phase Room temp	Saline phase 37°C	Coomb's phase 37°C	Result

ANTIBODY SCREENING

The testing of donor serum for unexpected blood group antibodies is required because these antibodies adversely affect the red cells of recipients. Cell panels of known antigen specificity are available commercially. The range varies with each size of the panel. Important antibodies are covered in a three-cell panel.

Procedure
1. Place three test tubes in a rack and label them as I, II and III.
2. Add 2 drops of patient's serum in each tube.
3. Add 1 drop of commercial phenotyped red cells from each vial to corresponding test tube.
4. Add 2 drops of LISS. Incubate at 37°C for 10 min.
5. Wash three times with normal saline.
6. Add 2 drops of Coomb's serum and centrifuge for 15 seconds at 3400 RPM.
7. Look for agglutination

Interpretation
Agglutination indicates the presence of antibody. Results are interpreted according to the sheets available with the commercially prepared red cell panels.

ANTIBODY SPECIFICITY

For the purpose of identification of specificity of the antibody detected in screening larger cell panels with known specificity are required. Most commonly used is the commercially available 11 cell panel.

Procedure
1. Place 11 test tubes in a rack and label them from 1 to 11.
2. Add 2 drops of patient's serum in each tube.
3. Add 1 drop of commercial cells from each vial to corresponding test tube.
4. Add 2 drops of LISS. Incubate at 37°C for 10 min.
5. Wash four times with normal saline.
6. Add 2 drops of Coomb's serum and centrifuge for 15 seconds at the rate of 3400 RPM.
7. Look for agglutination

Interpretation
Read the antibody specificity from the manufacturer's chart provided with the panel.

SCREENING FOR HAEMOLYSINS

Blood group O contains anti-A and anti-B antibodies, which may be haemolysins. When such blood having a high titre of these antibodies is transfused to persons of blood group A, B, or AB it may induce haemolysis of recipient's red cells. Such group O blood units are designated **dangerous universal donor**. This blood must be identified and used only for O group recipients. A sticker reading '**For O group recipients only**' must be applied on such a bag to avoid using it for non-group O recipients.

Procedure
1. Take two test tubes and label them as A and B.
2. Add 2 drops of donor serum in each test tube.
3. In test tube A add one drop of known A cell suspension.
4. In test tube B add one drop of known B cell suspension.
5. Keep both test tubes at 37°C for 2 hours.
6. Centrifuge and examine for evidence of haemolysis (pink colour of supernatant).

Interpretation
If there is haemolysis it means the blood is not safe and should not be given to other than group O recipients.

ANTIBODY ABSORPTION

Absorption is a process by which an antibody is allowed to react with antigen of the cell membrane to isolate it from the serum. The process is used for removing the unwanted antibodies from the serum for various purposes. It is also used for antibody identification after elution and detection of weakly expressed antigens on red cells.

Procedure
1. Wash the cells in normal saline six to eight times.
2. Mix one volume packed cells with one volume serum.
3. Place the mixture in water bath at 37°C for warm antibody absorption or in refrigerator at 4°C for cold antibodies.
4. Incubate for 30 min.
5. Centrifuge at the rate of 3400 RPM for 10 min (the centrifuge cups should be pre-chilled to 4°C or warmed to 37°C depending upon the antibody involved).
6. Remove supernatant (serum) and test it for complete adsorption of antibody with cells carrying the antigen.
7. Further absorptions may be required, if the antibody is not completely removed.

ELUTION

Elution is the process by which an adsorbed antibody is broken down from the antigen antibody complex with the help of heat, alcohol, ether or acid so that the antibody is liberated. The technique is used for identification of certain antibodies. The eluate should be tested immediately. If it is to be stored then bovine albumin to a final concentration of 10 mg/100 ml should be added to protect the antibodies.

HEAT ELUTION

Heat elution is best suited for the investigation of ABO haemolytic disease of the newborn & elution of IgM antibodies from red cells.
1. Wash the red cells in saline (at least four washings).
2. Centrifuge at the rate of 3400 RPM for 5 min.
3. To the washed, packed cells add equal amount of saline.
4. Mix and agitate continuously in a water bath at 56°C for 10 min.
5. Centrifuge rapidly while still hot and remove the cherry red supernatant. This is the eluate.

ETHER ELUTION

Ether elution is suitable for investigation of a positive direct antiglobulin test associated with warm reactive (IgG) auto or alloantibodies.
1. Wash the red cells in saline (at least four washings).
2. Centrifuge at the rate of 3400 RPM for five min.
3. To the washed packed cells add equal amount of saline.
4. Add a volume of ether twice that of the packed red cells.
5. Shake the tube vigorously for one min by keeping the thumb and allowing release of vapours frequently.
6. Place at 37°C for 30 min, mixing frequently.
7. Centrifuge at the rate of 3400 RPM for five min, three layers will be formed, a top layer of clear ether, a middle layer of denatured red cell stroma and a bottom layer of haemoglobin stained eluate.
8. Remove the top two layers by aspiration and discard.

9. Centrifuge the eluate at high speed and transfer it into another tube.
10. The eluate can be tested immediately or stored frozen at -20°C.

TEST FOR COLD AGGLUTININS

Cold agglutinins are antibodies that react best at cold temperature. The following procedure is adopted for their detection/titration.

1. Place 11 test tubes in a rack and label them as 1/1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512 and 1/1024.
2. Add 2 drops of saline in each tube.
3. Add 2 drops of patient's serum in the first tube.
4. Mix well and transfer 2 drops from 1st tube to 2nd tube. Repeat this transfer to the last tube from which 2 drops are discarded.
5. Add 2 drops of a 5% suspension of pooled group O cells to each tube.
6. Mix gently and place the rack at 4°C overnight (not less than 6 hours).
7. Remove the rack, centrifuge and examine for agglutination.

Interpretation
Highest dilution showing agglutination indicates the titre of cold antibodies.

COMPLICATIONS OF BLOOD TRANSFUSION

Transfusion of blood and blood products are associated with certain risks and unfavourable effects. As a result blood products should only be administered when alternate forms of therapy do not exist or are less effective. The side effects can be classified as follows:

1. IMMUNOLOGICAL
    a. Due to red cell antibodies
        i) Sensitisation to red cell antigens
        ii) Haemolytic transfusion reactions (Immediate and delayed)
    b. Due to white cell antibodies
        i) Febrile transfusion reactions
        ii) Transfusion related acute lung injury (TRALI)
        iii) Transfusion associated graft versus host disease (TA GVHD)
    c. Due to platelet antibodies
        i) Platelet refractoriness
        ii) Post-transfusion purpura
    d. Due to plasma protein antibodies
        i) Urticaria
        ii) Anaphylaxis
2. NON IMMUNOLOGICAL
    a. Transfusion transmitted infections
        i) Viral
            (1) Hepatitis B Virus(HBV)
            (2) Hepatitis C Virus(HCV)
            (3) HIV 1 and 2
            (4) Cytomegalovirus (CMV)
            (5) HTLV I and II
            (6) Parvovirus B_{19}
            (7) Epstein Barr (EB) virus
        ii) Protozoal
            (1) Malaria (Plasmodium spp.)
            (2) Chaga's disease (*T.cruzi*)
        iii) Bacterial
            (1) Syphilis (*Treponema pallidum*)
            (2) Yersinia enterocolitica
            (3) Pseudomonas spp.
            (4) Citrobacter, *E.coli*
            (5) Others
        iv) Prions
            (1) CJD (Creutzfeld-Jakob disease)
    b. Circulatory overload
    c. Citrate toxicity
    d. Potassium toxicity
    e. Acid overload
    f. Thrombophlebitis
    g. Air embolism
    h. Transfusion haemosiderosis
    i. Complications of massive transfusion
        i) Dilution of coagulation factors
        ii) Dilutional thrombocytopenia
        iii) Hyperkalaemia
        iv) Hypocalcaemia

INVESTIGATION OF HAEMOLYTIC TRANSFUSION REACTION

The following tests should be carried out in case of any untoward reaction after transfusion of blood.

1. **Bedside check**: Immediately check all the issue forms, blood bag and patients identification. Record and inform if any discrepancy is found.
2. **Check for haemolysis**
    a. Examine patient's plasma and urine for haemoglobin.
    b. Blood film may show spherocytosis, or agglutination.
    c. Biochemical evidence, including bilirubin and haptoglobin levels.
3. **Check for incompatibility**
    a. Clerical errors: An identification error will indicate the type of incompatibility. Recheck the particulars of patient on requisition form, pretransfusion cross match sample and post transfusion sample.
    b. Serological workup:
        i) Repeat ABO and Rh D group of

patient (pre and post transfusion samples) and donor unit.
   ii) Screen patient's serum (pre and post transfusion) for red cell antibodies.
   iii) Repeat cross match with pre and post transfusion serum.
   iv) Direct antiglobulin test (pre and post transfusion samples).
   v) When direct antiglobulin test is positive, elute the antibody from the cells.
4. Check for disseminated intravascular coagulation (DIC)
   a. Blood film (red cell fragmentation)
   b. Platelet count
   c. Coagulation screen
5. Check for bacterial infection
   a. Gram stain and culture donor and recipient's blood.
6. Check for baseline renal function (urea, creatinine, electrolytes).

SPECIAL TRANSFUSION SITUATIONS

There are some situations where the provision of compatible blood requires special consideration.

Compatibility tests in newborn infants

For infants under 4 months, both baby and maternal blood samples should be ABO and Rh D grouped, the maternal serum screened for atypical antibodies and a DAT done on the baby's cells. If a maternal antibody screen is negative and the baby's DAT is negative, blood of the same ABO and D group as the infant may be issued without cross matching, even when repeated small volume transfusions are required. Infants under the age of 4 months do not make red cell alloantibodies even after multiple small volume transfusions. Haemolytic disease of newborn: Haemolytic disease of newborn is defined as decrease in red cell survival of the infant due to presence of antibodies derived from the mother. These antibodies are IgG antibodies and cross the placenta to enter in the foetal circulation. They are produced in response to transplacental haemorrhage during pregnancy, in which the foetal red cells carrying antigens not present in the mother stimulate the maternal immune system. The most common antibody detected in haemolytic disease of newborn is anti D, followed by anti c and rarely anti K. Anti A or anti B in group 'O' mothers may have IgG component and may result in ABO haemolytic disease of newborn. With advanced diagnostic methods it is possible to detect haemolytic disease of newborn during pregnancy, and foetal exchange transfusions carried out using O Rh D negative fresh blood (Hct >70%), which is leuco-depleted and irradiated prior to transfusion. The following serological procedures are carried out in the laboratory in order to select appropriate blood for transfusion:

1. **Mother's sample**
   a. ABO and Rh 'D' grouping
   b. Antibody screening & identification
2. **Infant sample**
   a. ABO and Rh 'D' grouping
   b. Direct antiglobulin test
   c. Identification of antibody from eluate (if required)
3. **Cross match**:
   a. Maternal serum to be used.
   b. Donor blood unit compatible with mother and infant blood group
   c. If in doubt select O negative blood suspended in AB plasma
   d. If mother's serum is not available, use infant's serum/eluate from red cells

In ABO haemolytic disease of newborn always use group 'O' blood preferably suspended in AB plasma. This is because the corresponding maternal antibody is going to cause rapid haemolysis, if adult A or B cells are used for exchange transfusion. The appropriate blood required in haemolytic disease of newborn (other than ABO haemolytic disease) is shown in Table 42.7.

Table 42.7: Haemolytic disease of newborn (cross match policy).

MOTHER'S GROUP	BABY'S GROUP	DONOR GROUP
O	O	O
O	A	O
A	O	O
O	B	O
B	O	O
A	B	O
B	A	O
AB	AB	AB
AB	A	A
AB	B	B
A	AB	A
B	AB	B

Compatibility tests for intra-uterine transfusion

Blood for an intra-uterine transfusion should be tested for compatibility with the mother's serum. It should be group O, Rh D negative and K negative. It is essential to repeat the antibody identification on each fresh sample of the mother's serum to identify any new alloantibodies formed. Blood for intra uterine transfusion should be less than 5 days old. It

should be CMV seronegative, have a Hct <0.75, be irradiated to a minimum of 25 Gy to avoid graft versus host disease and transfused within 24 hours of irradiation. Plasma reduced blood or washed red cells suspended in saline should be used.

AUTOMATION IN BLOOD BANKING

The increase in workload and the requirement of reliability of tests has resulted in introduction of various automated serological procedures in the blood bank. These include automation in blood grouping, antibody screening, anti D quantitation and viral screening of blood donations by ELISA systems. Various machines for this purpose are designed for large workloads and not suitable for a normal hospital based blood bank. Some of the techniques and methods used in automated systems for blood grouping and red cell serology are as follows:

1. Individual reaction wells: In this the anti sera and the red cells are mixed in reaction wells, centrifuged and remixed. The results are read by photometric method. Examples include Kontron Groupamatic Systems.
2. Microplate method: Several systems are available, in which the serological reactions are carried out in microplates.
3. Continuous Flow Systems: In this the antisera react with the red cells in a continuous system of coils. Technicon Autogrouper utilises this technique, which is then interfaced with computer for recording of results.
4. Gel microcolumns: This includes interaction of antisera and red cells in solid phase Sephadex columns. Special centrifuge is required for the centrifugation of cards holding specific number of columns. This technique has the advantage that it is more reproducible and does not require any washing step. Examples include DiaMed and Diana Gel systems.

All the automated equipments are interfaced with computers and printers for recording of the results. It should be emphasised that introduction of automation in the laboratory will require more stringent quality control procedures and closer monitoring and maintenance. Hence, each laboratory should have critical analysis of costs and benefits of any such system, before introducing them as a routine.

Figure 42.1: Flow chart for Blood component preparation.

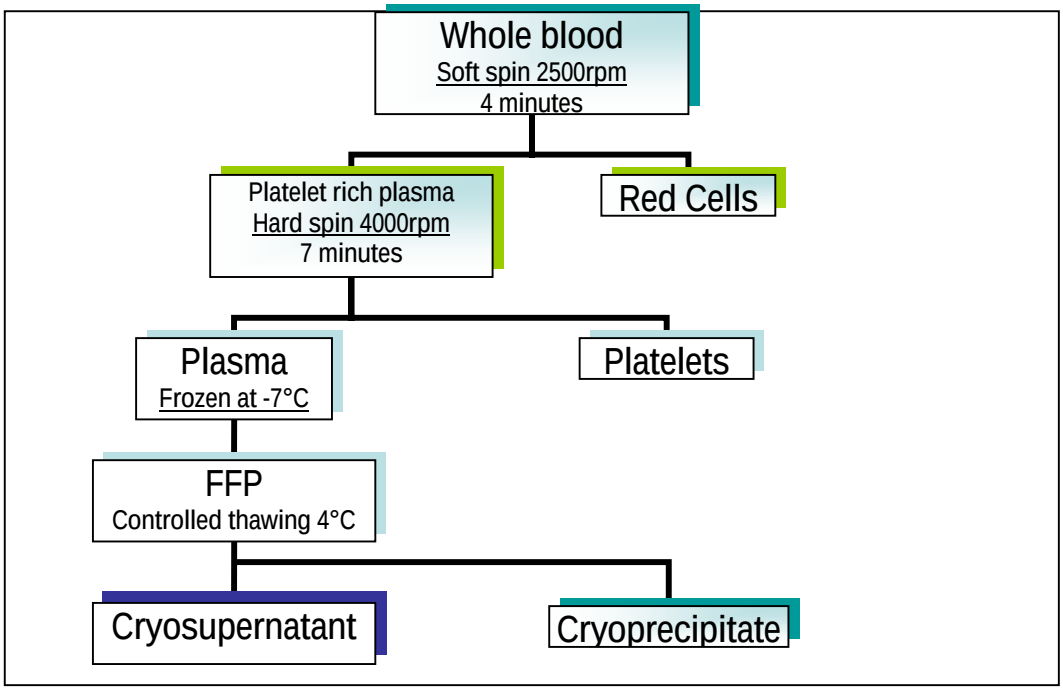
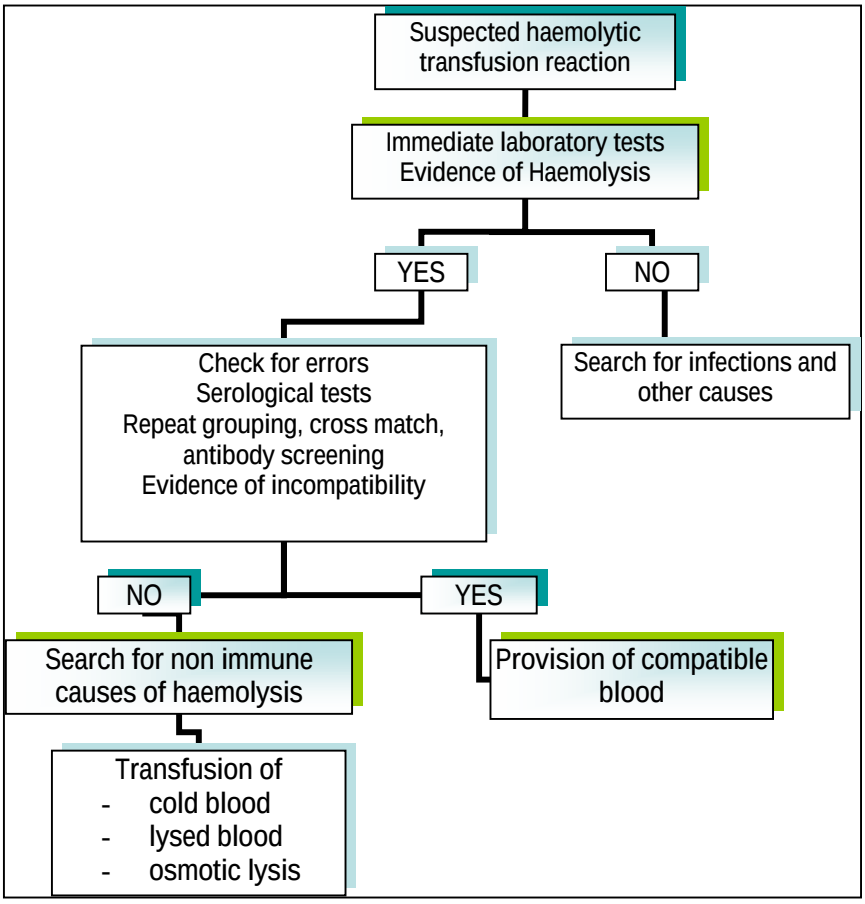


Table 42.8: Storage requirements and other information of various blood components

	Fresh Frozen Plasma	Cryoprecipitate	Red cell concentrate	Platelet concentrate
Preparation	Fresh plasma rapidly flrozen to -30°C	Thawing FFP unit at 4±2°C	Whole blood, centrifugation	Whole blood <8 hours
Volume	150-275 ml	20±5 ml	280±60 ml	50±10 ml
Contents	All coagulation factors, FVIII 200 units, Fibrinogen 250-400 mg/ unit	FVIII, vWF, Fibrinogen, FXIII	Hct: 0.55-0.75 l/l	Platelet count $5.5x10^{10}$/unit, erythrocyte count $<1x10^{9}$/unit, Stable factors, FV,FVIII
Storage	≤-30°C	≤-30°C	4±2°C	22±2°C
Shelf life	>12 months	>12 months	CPDA-1: 35 days, RCC in AS-1 42 days	5 days, pooled platelets must be used within 4 hours
Thawing	At 37°C water bath within 15-30 minutes	At 37°C water bath for 15 minutes, do not warm.	-	-
Administration	Through filter, without cross match	Through filter, within 2-6 hours, pooled precipitate within 4 hours	Through 170 µm filter,	≥19 gauge needle, 170 µm filter
Dosage	10 ml/kg body weight	One conc/5kg body weight	-	Increment 5000/unit concentrate
Rate of infusion	Within 4 hours	10 ml/minute as loading dose	Within 2-4 hours	10 minutes/unit
Required level	-	Minor bleed: 10-50% of factor, Major surgery: 80-100%, Post-op: >50% for 10-14 days	-	Corrected count increment (CCI) >7.5
Turn around time	-	-	30-45 minutes	-
Holding time	-	-	Normally: 24 hours, Exceptionally: 72 hours	-
Caution	May transmit disease	May transmit disease	Avoid if signs of deterioration obvious May transmit disease	May transmit disease
Demand type	-	-	Routine: ABO-Rh compatible Emergency: ABO type specific without cross match Disparate situation: O Rh negative without cross mach	Single unit platelets Pooled platelets
Avoid simultaneous administration of	-	-	Glucose solutions, lactate ringer, dextrose, dextrose saline, any other hypotonic solution, calcium, etc Any medication	-

Figure 42.2: Flow diagram for investigation of suspected transfusion reactions



SECTION VII - CHEMICAL PATHOLOGY, ENDOCRINOLOGY AND TOXICOLOGY

43. DIAGNOSTIC METHODS IN DIABETES MELLITUS

GLUCOSE METABOLISM

1. Glucose is the main product of dietary carbohydrate metabolism.
2. After a carbohydrate-containing meal excess glucose is:
   - stored as glycogen in liver and muscle;
   - converted to fat and stored in adipose tissue (Figure 43.1).

   Insulin stimulates these processes. The brain is almost entirely dependent on extracellular glucose as an energy source. Maintenance of plasma glucose concentration is important for normal cerebral function.
3. During fasting:
   - glycogen breakdown in the liver and, to a lesser extent, in the kidneys releases glucose into the plasma;
   - triglyceride breakdown in adipose tissue releases glycerol and fatty acids, glycerol can be converted to glucose, and available fatty acids can be metabolised by most tissues other than the brain.

   The liver converts excess fatty acids to ketones, which can be used as an energy source by the brain and other tissues. If ketoacids formation exceeds the capacity of homeostatic mechanisms ketoacidosis may develop.
4. Anaerobic glycolysis produces lactic acid:
   - lactic acid production occurs temporarily in contracting muscles;
   - the hypoxic liver becomes a major lactic acid producing rather than a lactic acid consuming organ, and lactic acidosis results. Other factors may also cause lactic acidosis by increasing glycolysis or by reducing the utilisation of lactic acid.

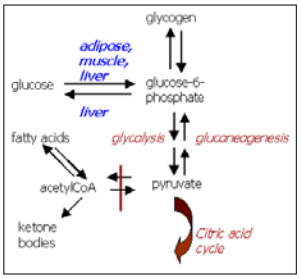


Figure 43.1: Outline of glucose metabolism

HYPERGLYCAEMIA

Hyperglycaemia may be due to:

- Diabetes mellitus;
- Intravenous infusion of glucose containing fluids;
- Severe stress such as cerebrovascular accidents (stress hyperglycaemia)

DIABETES MELLITUS

The term diabetes mellitus describes a metabolic disorder of multiple aetiology, characterised by hyperglycaemia with disturbances of carbohydrate, protein and fat metabolism, resulting from defects in insulin secretion, insulin action or both. The chronic hyperglycaemia of diabetes is associated with long term damage, dysfunction and failure of various organs especially the eyes, kidneys, nerves, heart and blood vessels. Several pathogenic processes are involved in the development of diabetes. These include processes, which destroy the β-cells of pancreas with consequent insulin deficiency, and others that result in resistance to insulin action. The abnormalities of metabolism are due to deficient action of insulin on target tissues resulting from insensitivity or lack of insulin. Classical symptoms of diabetes mellitus are polyuria, polydipsia, weight loss, sometimes with polyphagia and blurred vision. Impairment of growth and susceptibility to infections may accompany chronic hyperglycaemia. Acute life threatening consequences of diabetes are hyperglycaemia with ketoacidosis, the non-ketotic hyperosmolar syndrome, hypoglycaemia and rarely, lactic acidosis.

CLASSIFICATION OF DIABETES MELLITUS

In 1999 American Diabetic Association (ADA) in collaboration with WHO produced a revised classification of diabetes based on aetiology rather than clinical manifestations and treatment. This aetiological classification includes:

I. Type 1 diabetes
   A. Immune mediated
   B. Idiopathic
II. Type 2 diabetes
III. Other specific types
   A. Genetic defects of β-cells function
   B. Genetic defects in insulin action
   C. Diseases of exocrine pancreas
   D. Endocrinopathies
   E. Drugs or chemical induced

F. Infections
G. Uncommon forms of immune-mediated diabetes mellitus
H. Other genetic syndromes sometimes associated with diabetes mellitus
    1. Down's syndrome
    2. Klinefelter syndrome
    3. Turner's syndrome

IV. Gestational Diabetes Mellitus (GDM)

DIAGNOSTIC CRITERIA

In 1997 International Expert Committee working under the sponsorship of ADA introduced new diagnostic criteria for diabetes mellitus. Any one of the following is diagnostic:

1. Symptoms of diabetes plus random (casual) plasma glucose concentration of >11.1 mmol/L
2. Fasting plasma glucose (FPG) >7.0 mmol/L
3. Two hour plasma glucose level (post-glucose load, 2-hPG) >11.1 mmol/L during an OGTT

The diagnosis must be confirmed subsequently by any one of the above-mentioned criteria.

IMPAIRED FASTING GLUCOSE (IFG)

When FPG level is between 6.1-7.0 mmol/L.

ORAL GLUCOSE TOLERANCE TEST (OGTT)

It is an acceptable diagnostic test but is not recommended for routine use because it is inconvenient for the patient, costly, time consuming and has poor reproducibility.

Indications

OGTT is only indicated in the following conditions:

1. Diagnosis of Gestational Diabetes Mellitus (GDM)
2. When fasting plasma glucose is between 6.1-7.0 mmol/L or 2 h postprandial glucose levels are between 7.8-11.1 mmol/L.
3. Evaluation of patients with unexplained nephropathy, neuropathy or retinopathy with random (casual) plasma glucose more than 7.8 mmol/L

Preparation of Patient

1. The patient must be ambulatory and free from pyrexia, acute illness or trauma for at least two weeks.
2. He should have diet containing at least 150 g carbohydrate/day for three days prior to test.
3. Any drug that alters blood glucose level should also be discontinued for three days prior to testing (e.g., salicylates, steroids, thiazide diuretics, anticonvulsants). If patient is already under treatment with hypoglycaemic drugs e.g., insulin or the sulfonylureas, these should be discontinued at least on the day of the test.
4. To avoid circadian variation and to obtain a greater reproducibility the test should be done in the morning between 7 am to 9 am
5. Patient must have 8-16h fast. An average 12h fast is recommended
6. Heavy tea and coffee drinker should reduce their consumption during the day preceding the test
7. Smoking is not allowed during fast or at least in the morning before OGTT and during the OGTT.
8. No physical exercise is allowed during the test.
9. Patient should be seated quietly and relaxed for 30 min before the test.
10. If not already done, it is advisable to determine the patient's fasting plasma glucose level prior to OGTT. In case a definite hyperglycaemia exists, glucose load is contraindicated.

Procedure

WHO expert committee has recommended 75 g glucose load for the adults and 1.75 g/kg body wt up to a maximum of 75 g glucose for the children. Glucose is mixed in water (25 g/100 ml) and patient should drink it within 5 min. This is zero time. The second blood sample is taken at 2h. A 5h oral glucose tolerance test is needed for the diagnosis of reactive hypoglycaemia.

Factors affecting glucose tolerance

There are so many factors which can affect and disturb the glucose tolerance of an individual resulting in poor reproducibility of OGTT and difficulties in interpretation.

1. **Diet**: Low carbohydrate and low caloric diet reduces glucose tolerance. Impaired glucose tolerance has been observed in persons who have restricted their carbohydrate intake in anticipation of the test. Therefore, the diet should contain at least 150g carbohydrates daily for three days prior to the test. Extra tea and coffee should also be avoided during the days preceding the test.
2. **Physical activity**: Physical inactivity impairs the glucose tolerance.
3. **Intercurrent disease and injury**: Acute illness and trauma cause physical stress resulting in stress hyperglycaemia and reduced glucose tolerance. It is recommended that OGTT should not be performed at least 1-2 months after recovery

from acute myocardial infarction, trauma, burns and operations etc.

4. **Psychological stress**: Glucose tolerance test should not be done after a major emotional disturbance as this also results in stress hyperglycaemia.
5. **Endocrine diseases**: Most of the endocrine hypersecretory conditions impair glucose tolerance. Hyperthyroidism and pheochromocytoma are well known for this. The anti-insulin action of some of the hormones makes it impossible to interpret OGTT until the associated endocrinopathies has been adequately treated.
6. **Pregnancy**: Placental hormone production, particularly after the first trimester, decreases insulin sensitivity resulting in hyperglycaemia.
7. **Drugs**: Glucocorticoids and thiazide diuretics decrease glucose tolerance. On the other hand oral hypoglycaemic agents and salicylates improve glucose tolerance. It is a good practice to ovoid all medication at least 3 days before the test.

Interpretation

- **Normal** – 2h PG levels <7.8 mmol/L
- **Impaired glucose tolerance (IGT)** - 2h PG levels between 7.8-11.1 mmol/L
- **Diabetes mellitus** – Fasting glucose levels above 7.8 mmol/L or 2h PG levels >11.1 mmol/L

GESTATIONAL DIABETES MELLITUS GDM

It is defined as any degree of glucose intolerance with onset or first recognition during pregnancy.

- **Low risk group**: The low risk group comprises women who are <25 years of age and of normal body weight, have no family history of diabetes, have no history of abnormal glucose metabolism or poor obstetric outcome, and are not members of an ethnic/racial group with a high prevalence of diabetes. These women only require screening for GDM during 24-28 weeks of gestation.
- **High-risk group**: Women with clinical characteristics consistent with high risk of GDM (marked obesity, personal history of GDM, glycosuria, or a strong family history of diabetes) are directly subjected to OGTT with 100g glucose, not to the screening test.

SCREENING (GLUCOSE CHALLENGE TEST, GCT)

Fifty-gram glucose load is given at any time of the day without any prior preparation or fasting. Blood sample is collected at 1 hour for glucose. If plasma glucose is >7.8 mmol/L the patient is subjected to OGTT with 100g glucose load.

DIAGNOSIS (OGTT WITH 100g GLUCOSE LOAD)

It is performed in the morning after 8 to 14 hour fast. After measuring fasting glucose the patient is given 100 g glucose orally. This is zero time. Plasma glucose is subsequently measured hourly for 3 hours. Any two values exceeding the following confirm the diagnosis.

Fasting	5.8 mmol/L
1 h	10.5 mmol/L
2 h	9.1 mmol/L
3 h	8.0 mmol/L

If results are normal in a clinically suspected lady, OGTT is repeated after 4 weeks. Women with GDM are again evaluated 6-12 weeks postpartum. If glucose concentration returns to normal the patient is followed up as per high-risk group for diabetes mellitus. About 60% of women with gestational diabetes become overtly diabetic within 15 years.

STRESS HYPERGLYCAEMIA

Hyperglycaemia as a result of stressful conditions is a commonly encountered problem in a wide variety of clinical settings. Raised levels of stress hormones e.g., epinephrine, cortisol, growth hormone and glucagon are responsible for hypercatabolism and elevation of plasma glucose levels. Degree of hyperglycaemia varies from mild to severe with no upper limit, and it disappears once the stress is over. It has to be differentiated from diabetes mellitus by glycated haemoglobin and fructosamin levels. Inability to do so may result in over enthusiastic diagnosis of diabetes mellitus.

HYPOGLYCAEMIA

Plasma glucose concentration <2.5 mmol/L (45 mg/dl) collected in a tube containing fluoride is defined as hypoglycaemia. Symptoms are due to sympathetic activity such as sweating, tachycardia, agitation and headache. Patients on β-blockers or those with peripheral neuropathy (long term complication of diabetes mellitus) may not show these symptoms. Other symptoms are faintness, dizziness, lethargy and ultimately leading to coma and death if not treated promptly. The hypoglycaemia may be:

- **Fasting** when symptoms occur typically at night or in the early morning, or may be precipitated by a prolonged fast or strenuous exercise. This pattern suggests excessive

utilisation of glucose or an abnormality of the glucose sparing or glucose forming mechanisms.

- **Non-fasting** when symptoms occur within 5-6 hours after a meal and may be related to ingestion of a particular type of food, or be associated with medication. Substances that may provoke hypoglycaemia are summarised in Table 43.1. Reactive and drug induced hypoglycaemia are the commonest causes in adults. Fructose or leucine ingestion are important causes in infants (Table 43.2).

Table 43.1: Principal causes of fasting hypoglycaemia in adults

Inappropriately high insulin concentrations due to:
Pancreatic tumour
Hyperplasia of the pancreatic islet cells
Glucocorticoids deficiency
Sever liver disease
Some non pancreatic tumours
Drugs
Insulin
Sulphonylureas
Alcohol
Glucose (reactive hypoglycaemia)
Fructose (in sucrose containing foods)
Leucine (an amino acid in casein, a protein in milk

Table 43.2: Principal causes of hypoglycaemia in infants and children

Neonatal period
'Small for dates' infants
Hypoxia at birth
Infants of diabetic mothers
Erythroblastosis foetalis (rare)
Early infancy
Endocrine causes
Hypopituitarism
Adrenal insufficiency
Inborn errors of metabolism
Glycogen storage diseases, such as von Gierkes's disease
Hereditary fructose intolerance
Lat infancy
Ketotic hypoglycaemia of infancy
Nesidioblasosis (islet cell hyperplasia)
Leucine sensitivity

PLASMA GLUCOSE ESTIMATION

SPECIMEN COLLECTION AND STORAGE

In individuals with normal haematocrit, fasting whole-blood glucose concentration is approximately 12% to 15% lower than plasma glucose. In most clinical laboratories, plasma is used for glucose determination, whereas most methods for self-monitoring of glucose use whole blood. Glycolysis decreases blood glucose by approximately 5% to 7% per hour in normal uncentrifuged blood at room temperature. If separated, in non-haemolysed sterile serum or plasma the glucose concentrations are generally stable up to 8 hours at 25°C and up to 72 hours at 4°C because plasma has no glycolytic activity. Glycolysis can be inhibited and glucose stabilised for as long as 3 days at room temperature by addition of sodium fluoride (NaF) to the specimen. EDTA is used as an anticoagulant with NaF in commercially prepared glucose tubes.

METHODS OF GLUCOSE ESTIMATION

There are three types of methods commonly used for estimation of plasma glucose. These are:

1. Copper reduction methods e.g., Folin-Wu method
2. Other reduction methods e.g., Orthotoluidine method
3. Enzymatic methods e.g., Hexokinase, Glucose oxidase and Glucose dehydrogenase methods

Folin-Wu method[1]

Principle: It is a quantitative end point method. Proteins are precipitated by tungstic acid. Cupric ion (Cu^{+++}) in alkaline solution is reduced to cuprous ion (Cu^{++}) by glucose, which then reacts with phosphomolybdic acid to form blue coloured molybdenum. This is measured by colorimeter. Reoxidation of cuprous to cupric ion by atmospheric oxygen during the test procedure before addition of phosphomolybdic acid can occur. This can be avoided by using special narrow neck tubes called Folin-Wu tubes.

Orthotoluidine method

Principle: Protein is precipitated with trichloracetic acid (TCA). Orthotoluidine reacts quantitatively with the aldehyde group of aldohexoses to form a glycosylamine and Schiff base. A green compound is formed (glycosylamine + Schiff base) which is measured photometrically at 630 nm. The orthotoluidine reagent contains glacial acetic acid, which is highly corrosive. Orthotoluidine itself is carcinogenic, therefore, the test has been discontinued and has been replaced with more specific methods.

Hexokinase (HK) method

Principle: ATP phosphorylates glucose in the presence of hexokinase and Mg^{++}. The glucose phosphate formed is oxidised by G6-PD to 6-phsophogluconate in the presence of NADP. The amount of reduced NADP (NADPH) produced is directly proportional to the amount of glucose in the sample and it measures the

[1] This method is now obsolete.

change in absorbance at 340 nm. The method has very good accuracy and precision. It has been proposed as basis of reference method. Method can be automated.

Uses: Plasma, CSF and urine glucose estimation.

Glucose oxidase method

Principle: The enzyme glucose oxidase catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide (H_2O_2) as follows:

$$\text{Glucose} + 2H_2O + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + 2H_2O_2$$

Addition of enzyme peroxidase and a chromogenic oxygen acceptor, such as ortho dianisidine, results in the formation of a coloured compound that can be measured. The test is quantitative and can be performed in both kinetic and end point analysis mode. The test can be automated. It has good accuracy and precision.

Uses: Plasma and CSF glucose estimation.

Glucose dehydrogenase method

Principle: The enzyme glucose dehydrogenase catalyses the oxidation of glucose to gluconolactone. The amount of NADH generated in the reaction is proportional to the glucose concentration. The test is quantitative and can be performed in both kinetic and end point analysis mode. The test can be automated.

Uses: Plasma and CSF glucose estimation.

Reagents and Procedure: The reagents of above methods are available commercially. Their in-house preparation is not cost/labour intensive. Follow the detailed procedure as per standard instructions provided by the manufacturer of the commercial kit in use.

Conversion factor: See Table 2.6 on page 9.

GLYCATED HAEMOGLOBIN (HbA_{1c}) ESTIMATION

Glycation is the nonenzymatic attachment of glucose to amino acid residues of haemoglobin. In normal individuals with normal blood glucose level, 6-8% haemoglobin is glycated. However hyperglycaemia promotes increased nonenzymatic glycosylation of haemoglobin, the measurement of which is used to assess diabetic control. Techniques available for the estimation of HbA_{1c} include:

- Electrophoresis
- Colorimetric determination
- Ion exchange chromatography
- Radioimmunoassay
- Affinity chromatography

Ion-exchange chromatographic method

Principle: HbA and other haemoglobin fractions contained in whole blood haemolysate are introduced on to a cation exchange resin column. HbA and other haemoglobins are absorbed onto the ion exchange material. Addition of a known volume of eluting solution to the column removes only HbA_1. Measurement of the absorbance of this eluate and of the original haemolysate at 415 nm permits quantitation of the HbA_1 fraction.

Procedure and reagents: Commercial kits for the determination of glycated Hb are available from “Sigma diagnostics”, “Human” etc. The user is advised to follow the instructions of kit procedure strictly for good results.

Advantages: Ion exchange chromatographic method is precise and accurate and is most commonly used.

Disadvantages: The method is quite expensive and may be temperature dependent.

Sources of Analytical Errors

1. Insufficient washing of RBCs.
2. Pipetting errors.
3. Interfering substances.
4. Time factor and temperature also affect the accuracy.

URINARY GLUCOSE

Glycosuria is defined as a concentration of urinary glucose detectable using relatively insensitive but specific screening tests. These tests depend on the action of the enzyme, glucose oxidase, incorporated into a diagnostic strip. Usually the proximal tubular cells reabsorb most of the glucose in the glomerular filtrate. Although very low urinary glucose concentrations may be detectable by more sensitive methods even in normal subjects, glycosuria, as defined above, occurs only when the plasma and therefore glomerular filtrate concentrations, exceed the tubular reabsorptive capacity, this may be because:

- The plasma and glomerular filtrate concentrations are more than about 11 mmol/L, and therefore the normal tubular reabsorptive capacity is significantly exceeded.
- The tubular reabsorptive capacity is reduced, as for example during pregnancy, so that glycosuria occurs at a lower filtrate concentration (**renal glycosuria**). This is usually a harmless condition.

Very rarely if the GFR is much reduced, there may be no glycosuria despite plasma glucose concentrations above 11 mmol/L. if the volume of glomerular filtrate is very low the total amount of glucose delivered to tubular cells may be less than normal, even if the concentration is high. In such rare cases urine testing cannot be used to

monitor diabetic control. Glycosuria should be sought in a urine specimen produced by the kidneys, collected about an hour after a meal, when peak plasma concentrations are reached by the double void technique. This ensures that the specimen being tested reflects the plasma glucose concentration at the same time and has not been stored in the bladder for sometime. Prior to collection; specimens collected after a period of fasting yield positive results only when fasting plasma glucose concentrations are above about 11 mmol/L or during infusion of glucose or if there is gross renal glycosuria. Reducing substances in the urine can be detected using copper containing reagents (Table 9.4). For details see chapter on URINE EXAMINATION on page 80 in the section on Clinical Pathology.

MICROALBUMINURIA

Sensitive immunological assays have shown the normal daily excretion of albumin to be <20 mg. Patients with diabetes mellitus who excrete between 20-300 mg/L are said to have microalbuminuria and to be at greater risk of developing progressive renal disease than those whose albumin excretion is normal. The incidence of this complication may be reduced by more stringent control of plasma glucose concentration and blood pressure. See also page79.

44. LIVER FUNCTION TESTS

Liver in a healthy adult weighs about 1500 g. It is composed of numerous lobules, each being an independent structural and functional unit. Each lobule comprises of a central vein and hepatocytes, arranged in the form of columns of cells around the central vein. On one side of these columns are the sinusoids, the dilated venous channels lined by endothelial cells, and cells of reticuloendothelial system. Interstitial spaces exist between the endothelial cells and hepatocytes, known as the space of Disse. On the other side of hepatocytes are the bile canaliculi in which bile flows to the bile duct. Portal triad is the structure present at the junction of lobules and contains hepatic artery, portal vein and bile duct. Liver is an essential organ of the body, which performs a wide range of excretory, synthetic, storage, detoxification and filtration functions. Briefly these are:

1. **Metabolism of carbohydrates, lipids and proteins**: Portal blood, which is rich in all the absorbed nutrients, except fat, enters the liver where glucose is taken up and converted into glycogen or fatty acids. Blood glucose level in the fasting state is maintained by conversion of this glycogen back to glucose.
2. **Synthetic function**: A number of proteins present in the plasma including albumin, globulin, fibrinogen and other coagulation system proteins are synthesised in liver. These proteins perform different functions. Plasma oncotic pressure, which prevents loss of fluid into tissue spaces, is because of plasma proteins, mainly albumin. Thus in advanced liver disease when albumin synthesis is impaired, this capability is lost and generalised oedema occurs. Albumin also acts as a ready source of amino acids whenever required. Coagulation factors, except VIII C are synthesised in liver. Factor II, V, VII, IX, and X require Vitamin K for their synthesis, the absorption of which is dependent on the presence of bile in the small intestine.
3. **Bilirubin Metabolism**: Bile salts and bilirubin are excreted in the bile. Bilirubin is mainly (80%) derived from the destruction of RBCs in RE system and about 20% is derived from other non-haem sources. In liver it is conjugated with glucuronic acid to make it water-soluble, is then excreted through urine, and is responsible for normal pale yellow colour of urine. Partly it is re-excreted in bile.
4. **Excretion and detoxification**: The liver excretes bilirubin and detoxifies certain drugs before they are eliminated from the body. Steroids are inactivated by conjugation as glucuronides and sulphates before excretion in urine. Steroid hormones produced by the body itself are also detoxified in liver. Amino acids are deaminated in the liver. Cholesterol is excreted in the bile either unchanged or after conversion to bile acids.
5. **Filtering function**: The Kupffer cells remove certain toxic substances coming from portal circulation before they enter the general circulation.

Derangement of liver functions, singly or in combination, may occur when liver is assaulted by:

- Viruses
- Drugs
- Industrial chemicals
- Hypoxia due to shock, congestive cardiac failure
- Prolonged biliary obstruction

In addition, the disease process also destroys the liver cells and this causes leakage of intracellular enzymes into plasma where their level rises.

JAUNDICE

Jaundice is the yellow coloration of sclera and other tissues because of accumulation of bilirubin in the body. It is the commonest sign, which draws attention to liver disease. However in a number of cases it may not be due to liver disease at all. Bilirubin is mostly produced by destruction of red blood cells. Liver only conjugates it and transfers it to bile through which it is excreted. Thus it may accumulate due to:

1. Increased destruction of red blood cells (haemolytic or pre-hepatic jaundice).
2. Reduced handling by liver cells (hepatic jaundice).
3. Reduced excretion due to obstruction of biliary passages (post-hepatic or obstructive jaundice).

Simple measurement of bilirubin in serum is not enough to find out the cause of jaundice. Other tests, including those of liver function, should also be performed to find out the real cause of jaundice. These test are:

1. **Tests of excretory function**
   Bilirubin (direct and indirect)
2. **Tests of liver damage**
   a. Aspartate transaminase (AST)
   b. Alanine transaminase (ALT)
3. **Tests of synthetic function**
   a. Total protein
   b. Albumin/Globulin ratio
   c. Coagulation factors (prothrombin)
4. **Tests of obstruction**
   a. Alkaline phosphatase
   b. γ-glutamyl transferase
   c. 5-Nucleotidase

SERUM BILIRUBIN ESTIMATION

Principle: Bilirubin reacts with diazotised sulphanilic acid to form an azo dye, which is red in neutral, and blue in alkaline solutions. Whereas the water-soluble bilirubin glucuronides react "directly", the free "indirect" bilirubin reacts only in the presence of an accelerator. The **total bilirubin** in serum is determined using the method of Jendrassik-Grof by coupling with diazotised sulphanilic acid after the addition of caffeine, sodium benzoate and sodium acetate. A blue azobilirubin is formed in alkaline Fehling solution This blue compound can also be determined selectively in the presence of yellow by-products (green mixed coloration) by photometry at 578 nm. The **direct bilirubin** is measured as the red azo dye at 546 nm using the method of Schellong and Wende without the addition of alkali. This method is based on the definition of direct bilirubin as that quantity of bilirubin, which, without the addition of an accelerator, can be determined after a reaction time of 5 min. This bilirubin comprises mainly the water-soluble bilirubin glucuronides. The **indirect bilirubin** is calculated from the difference between the total and direct bilirubin.

Reagents
Commercial reagent kits having all the necessary reagents packed together are available. However, these can be prepared as follows:

1. **Caffeine solution**: Dissolve 50 g caffeine, 75 g sodium benzoate, and 125 g crystalline sodium acetate trihydrate in warm water, cool and make up to 1 L. It is stable for 6 months.
2. **Diazo I**: Dissolve 5 g sulphanilic acid and 15 ml concentrated HCl in water to make 1 L.
3. **Diazo II**: Dissolve 0.1 g sodium nitrite in 20 ml water. Stable for several weeks.
4. **Diazo mixture**: Mix 10 ml diazo I and 0.25 ml diazo II. Stable for 24 hours at room temperature, and for 72 hours at 4°C.
5. **Hydrochloric acid 0.05 mol/L**: Dilute 4.5 ml concentrated HCl to 1 L.
6. **Alkaline tartrate**: Dissolve 100 g sodium hydroxide and 350 g sodium potassium tartrate and dilute to 1L. Store in a polythene bottle.

Procedure
Follow the detailed procedure as per standard instructions provided by the manufacturer of the commercial kit in use. For reagents prepared in the laboratory as described above, following procedure is followed:

1. Set up two tubes for each test, labelled as test and sample blank, and one tube as reagent blank for the whole batch.
2. Add 200 µl serum in tube marked as test and sample blank, and 200 µl water in the tube marked as reagent blank.
3. Add 2 ml caffeine solution to each tube.
4. Add 0.5 ml diazo mixture to test and reagent blank, and 0.5 ml diazo I to serum blank. Allow to stand for 10 minutes.
5. Add 1.5 ml alkaline tartrate to all the tubes, allow to stand for 5-10 minutes and read absorbance of test and sample blank against reagent blank at 600 nm. Subtract absorbance of sample blank from test and note the result from standard curve. To make a standard curve see PREPARATION OF CALIBRATION CURVE on page 47.
6. The result will be for total bilirubin. For direct bilirubin, omit the step of adding caffeine solution.

Reference range
Total bilirubin: 4-17.0 µmol/1
Direct bilirubin: ≤4 µmol/1
Conversion factor: See Table 2.6 on page 9.

Interpretation

1. **Physiological increase**
   a. Newborn.
   b. Unacclimatised people at high altitude
   c. Pregnancy
   d. Severe exercise
2. **Pathological increase**
   a. Unconjugated (Indirect)
      i) Haemolysis
      ii) Haemolytic disease of newborn
      iii) Hepatitis
      iv) Gilbert disease
      v) Crigler Najjar Syndrome

b. Conjugated (Direct)
   i) Liver damage due to any cause
   ii) Liver infiltration by tumour
   iii) Obstruction to biliary passages due to both intra and extra hepatic causes

3. **Pathological decrease**
   a. Long-term treatment with phenobarbitone

ALANINE TRANAMINASE (ALT) ESTIMATION

ALT is present in high concentrations in the liver and to a lesser extent in kidney, heart and skeletal muscle, pancreas, spleen and lung. Increased levels of ALT however are generally a result of liver disease associated with some degree of hepatic necrosis such as cirrhosis, carcinoma, viral or toxic hepatitis, whereas for most patients with chronic hepatic disease, ALT levels are generally lower than AST levels. Elevated ALT levels have also been found in extensive trauma and muscle disease, circulatory failure with shock, hypoxia, myocardial infarction and haemolytic disease.

Principle: The series of reactions involved in the assay system is as follows:

$$\text{L - Alanine} + \text{2 - Oxoglutarate} \xrightarrow{\text{ALT}} \text{Pyruvate} + \text{L - Glutamate}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LD}} \text{Lactate} + \text{NAD}$$

Specimen: Use non-haemolysed serum. Care should be taken in collection, transportation and processing of specimen to avoid all causes of haemolysis. Serum samples may be stored for at least 3 days at room temperature (18-25°C) and for at least 1 week at 4°C.

Reagents and procedure

1. Follow the detailed procedure as per standard instructions provided by the manufacturer of the commercial kit in use.
2. The reagent and sample volumes may be altered proportionally to accommodate requirements of different spectrophotometer and analysers' requirements.
3. If the change in absorbance is greater than 0.26/min repeat the assay after diluting with saline. Remember to adjust the final result by the dilution factor.
4. Valid results depend on an accurately calibrated instrument, timing, and temperature control.

Quality Control: To ensure adequate quality control, normal and abnormal control with assayed values should be run as unknown samples:

- When a new bottle of reagent is used.
- After preventive maintenance is performed or a critical component is replaced.
- With a batch of test samples (or every eight hours in case of fully automated analysers).

Control results falling outside the upper or lower limits of the established ranges indicate the assay may be out of control. The following corrective actions are recommended in such situations:

- Repeat the same controls if repeated control results are outside the limits, prepare fresh control serum and repeat the test.
- If results on fresh control material still remain outside the limits, then repeat the test with fresh reagent.

Limitations: The reagent contains LD to rapidly reduce endogenous sample pyruvate during the initial incubation time. Abnormally high levels of pyruvate may cause falsely high results (The normal level of serum pyruvate is 0.03 to 0.10 mmol/L).

Reference range

Adults: 10-42 U/L
Newborn/Infants: 7-40 U/L

Interpretation

1. Markedly raised levels
   a. Viral hepatitis
   b. Toxic liver necrosis
   c. Circulatory failure with shock and hypoxia
2. Moderately raised levels
   a. Cirrhosis (up to thrice normal)
   b. Cholestatic Jaundice
   c. Liver congestion secondary to cardiac failure
   d. Infectious mononucleosis with Liver involvement
   e. Extensive trauma and muscle disease (much less than AST)

ALKALINE PHOSPHATASE (ALP) ESTIMATION

Principle: Alkaline phosphatase catalyses the hydrolysis of *p*-nitrophenylphosphate, in the presence of magnesium ions, liberating inorganic phosphate and p-nitrophenol. The rate of p-nitrophenol formation is proportional to the concentration of ALP present in the sample.

$$\text{4 - nitrophenylphosphate} + \text{H}_2\text{O} \xrightarrow{\text{ALP (Mg}^{++}\text{)}} \textit{p}\text{ - nitrophenol} + \text{PO}_4$$

Specimen: Serum or heparinised plasma.
Stable for at least 7 days at 2-8°C.

Reagents and procedure: Follow the detailed procedure as per standard instructions provided by the manufacturer of the commercial kit in use.

Reference range

Adults: 65-306 U/L
Children: 185-625 U/L

Interpretation

Raised levels are seen in the following

conditions:

1. Physiological
   a. Children: until about the age of puberty two times adult normal
   b. Last trimester of pregnancy
2. Pathological
   a. Bone disease
      i) Osteomalacia and Rickets
      ii) Primary hyperparathyroidism
   b. Liver Disease
      i) Intra and extra hepatic cholestasis
      ii) Space occupying lesions
      iii) Granulomatous infiltration

Sources of non-analytical errors:

1. Heparinised plasma gives slightly lower (2-4%) results than serum.
2. Changes in posture or venous stasis will lead to changes in alkaline phosphatase.
3. It has been reported that results for alkaline phosphatase may be increased by as much as 25% in some subjects following food ingestion.
4. Ingestion of a high fat meal cause increase in alkaline phosphatase activity in subjects who are blood group O secretors.
5. Haemolysis interferes due to the high concentration of alkaline phosphatase in red cells.
6. Anticoagulants (fluoride, oxaloacetate, citrate and EDTA) inhibit alkaline phosphatase activity.
7. Frozen samples and reagents are to be brought to room temperature; otherwise the result will be spuriously high.
8. A number of drugs and substances are known to affect alkaline phosphatase values.

γ-GLUTAMYL TRANSFERASE (γGT) ESTIMATION

This enzyme was originally termed as transpeptidase. This enzyme causes the transfer of γ-glutamyl group from peptides and compounds that contain it, to other peptides or amino acids. This enzyme is present in all tissues except the muscle. It may act to transfer amino acids and peptides into the cells across the cell membrane in the form of γ-glutamyl peptide.

Principle: This enzyme catalyses the following reaction:

$$\gamma\text{ glutamyl-}p\text{-nitroanilide} + \text{Glycylglycine} \xrightarrow{\gamma GT} \text{Glutamylglycylglycine} + p\text{-Nitroaniline}$$

The increase in absorbance at 405 nm due to the *p*-nitroaniline formed in the reaction is measured photometrically.

Reference range: (Varies with different methods)

Males = 11-60 U/L
Females = 5-50 U/L.

Clinical significance: Even though renal tissue has the highest level of γGT, the enzyme present in serum appears to originate primarily from the hepatobiliary system. γGT activity is elevated in all forms of liver disease. It is highest in cases of intra- or post hepatic biliary obstruction reaching 5-30 times of normal levels. It is more sensitive than alkaline phosphatase, 5-nucleotidase in detecting obstructive jaundice, cholangitis and cholecystitis. Moderate elevations (2-5 times normal) are seen in viral hepatitis. Normal levels of γGT are seen in cases of skeletal disease. Thus measurements of γGT levels in serum can be used to ascertain whether observed elevations of alkaline phosphatase are due to skeletal disease or reflect the presence of hepatobiliary disease. Elevated values are observed in alcohol and drugs intake (Barbiturates).

PLASMA AMMONIA ESTIMATION

The major source of circulating ammonia is the gastrointestinal tract. Portal vein plasma ammonia is typically 5-10 fold higher than that in the general circulation and is derived from the action of bacterial proteases and urease on the contents of the colon. Under normal circumstances most of the portal vein ammonia load is metabolised to urea in liver cells.

Principle: The enzymatic method used is based on the following reaction:

$$2\text{-Oxoglutarate} + NH_4 + NADPH \xrightarrow{\text{Dehydrogenase}} \text{Glutamate} + NADP^+ + H_2O$$

The change in absorbance at 340 nm is measured as NADPH is transformed to $NADP^+$. The method is fast and simple.

Precautions:

1. The patient must not smoke after midnight when the fasting blood sample is drawn.
2. In order to minimise contamination of specimens and glassware by ammonia in the laboratory atmosphere, test should be done in isolated room.
3. Poor venepuncture technique may result in increased ammonia level.
4. Metabolism of nitrogenous constituents in the specimen is a source of ammonia contamination. The specimen must be put on ice immediately and centrifuged without delay.

Procedure: Follow the detailed procedure as per standard instructions provided by the manufacturer of the commercial kit in use.

Reference Range: Plasma: 11-35 μmol/L

Clinical Significance: Raised levels are seen in inherited deficiencies of urea cycle enzymes in

infants and in advanced liver disease.

SERUM TOTAL PROTEIN ESTIMATION

Principle (Biuret Method): Any compound (proteins) containing three or more peptide bonds reacts with alkaline copper tartrate reagent to form a blue to purple coloured substance. The intensity of colour produced is proportional to the number of peptide bonds reacting and, therefore, to the amount of proteins.

Reagents

1. Sodium hydroxide, 6 mol/L: Dissolve 240 g NaOH from a freshly opened bottle in water and make up to 1L with fresh distilled water.
2. Biuret reagent: Dissolve 3 g copper sulphate pentahydrate in about 500 ml water. Add 9 g sodium potassium tartrate and 5 g potassium iodide, add 100 ml 6 mol/L NaOH and make up to 1L with distilled water.

Procedure: Follow the detailed procedure as per standard instructions provided by the manufacturer of the commercial kit in use. For reagents prepared as above follow the procedure below:

1. Take two test tubes for test and blank. Add 5 ml Biuret reagent to both.
2. Add 100 µl sample to test and water to blank.
3. Mix and let stand for 30 min at room temperature.
4. Read absorbance of test at 540 nm against blank and note the result from calibration curve.

Reference range: Serum 65-80 g/L.

Interpretation

1. Total proteins may be increased in:
   a. Dehydration
   b. Hypergammaglobulinaemia
   c. Infections
2. Decrease in serum total proteins may occur in the following conditions:
   a. Chronic liver disease e.g., cirrhosis.
   b. Nephrotic syndrome
   c. Ascites.
   d. Cardiac failure
   e. Protein losing enteropathy

Physiological variations

1. Plasma volume decreases by 10-15% when the posture is changed from recumbent to upright, leading to alteration in the concentration of proteins.
2. A small increase in plasma volume may occur 2 hours after an 800 caloric meal and after exposure to heat.
3. Total protein concentration in heparinised plasma is approximately 0.3 g/dl higher than in serum because of the presence of fibrinogen in plasma.
4. The increase in serum protein after exercise is due to a decrease in blood volume.
5. Oral contraceptives and pregnancy decrease total protein concentration due to decrease in albumin.

SERUM ALBUMIN ESTIMATION

Principle:

Biuret method: Globulins in the serum are precipitated by 23% solution of sodium sulphate and ether. Remaining albumin is reacted with Biuret reagent to form a blue coloured compound, which is read in spectrophotometer at 530 nm.

Dye binding method: Albumin in the serum reacts with bromocresol green to give a green coloured compound the intensity of which is proportional to the amount of albumin present.

Reagent:

Colour reagent: Dissolve 8.8 g succinic acid in 200 ml water, add 85 mg bromocresol green already dissolved in about 5 ml 0.1 mol/L NaOH and dilute to about 800 ml with water. Add 1 mol/L NaOH to bring *p*H to 4.2 and make up to 1L with distilled water. Store at 4-8°C and pre-warm before use.

Procedure: Follow the detailed procedure as per standard instructions provided by the manufacturer of the commercial kit in use. For reagent prepared as above, use the following procedure:

1. Take two test tubes marked as test and blank.
2. To 4 ml reagent add 20 µl sample and water to test and blank respectively.
3. Read absorbance of test against blank after 30 seconds. It is better to zero the instrument before addition of sample to the reagent.
4. Determined the concentration of the test from calibration curve.

Reference range: Serum: 35-45 g/L

45. RENAL FUNCTION TESTS

FUNCTIONS OF THE KIDNEY

The functions of the kidney include following:

1. **The Excretory Function**: This means removal of most of the undesirable end products of the metabolism and any excess of inorganic substances of the diet. Waste products include urea, creatinine, uric acid and phosphates.
2. **The Regulatory Function**: It has a major role in homeostasis and includes:
   a. Acid-base regulation.
   b. Maintenance of fluid and electrolyte balance.
3. **The Endocrine Function**: In their primary endocrine function kidneys produce erythropoietin, renin and prostaglandins. Kidneys are also responsible for converting 25 hydroxycholecalciferol to 1,25 dihydroxy-cholecalciferol.

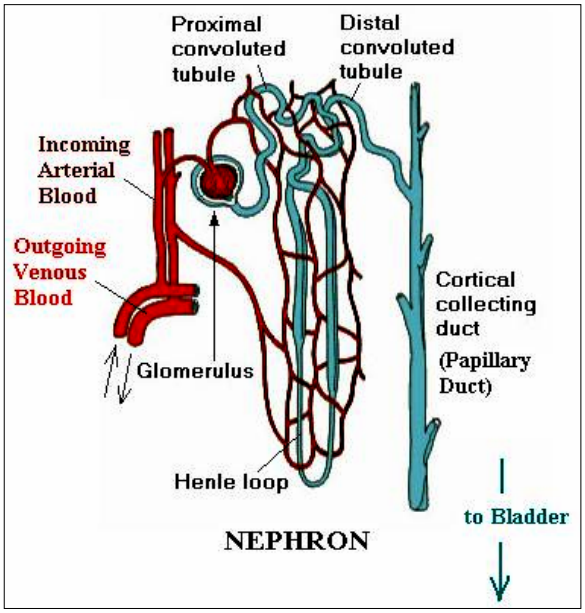


Figure 45.1: Different parts of a nephron.

BASIC UNIT OF THE KIDNEY

Nephron is the basic unit of renal function (Figure 45.1). There are about one million nephrons in each kidney. Each nephron has two parts:

1. **Glomerulus**: This is a spherical epithelial space with a capillary tuft inside. This is the site of first step of urine formation i.e. filtration of plasma water. The filtrate is called ultra-filtrate because it contains substances of molecular weight less than 15000.
2. **Tubules**: These are tubes of epithelial cells continuous with the glomerular epithelial space and ultimately leading to the collecting ducts and renal pelvis. In the tubules the process of reabsorption and secretion alters the composition of the ultra-filtrate.

PATHOPHYSIOLOGY

1. Normal renal function depends on a normal filtration rate and normal tubular function. A low glomerular filtration rate (GFR) leads to:
   a. Oliguria;
   b. Uraemia and retention of other nitrogenous end-products including creatinine and urate, and phosphate;
   c. A low plasma bicarbonate with metabolic acidosis;
   d. Hyperkalaemia
2. Tubular damage leads to:
   a. Polyuria. The urine is inappropriately dilute and contains an inappropriately high sodium concentration in relation to the patient's state of hydration;
   b. A low plasma bicarbonate concentration with metabolic acidosis;
   c. Hypokalaemia;
   d. Hypophosphataemia and hypouricaemia
3. In most cases of renal disease impairment of glomerular and tubular function coexist. The clinical findings depend on the proportions of each and on the total number of nephrons involved.
4. A low GFR without significant renal damage may be due to a reduced hydrostatic pressure gradient between the capillary plasma and the tubular lumen. This is most commonly due to renal circulatory insufficiency but may be caused by post-glomerular obstruction.
5. In acute oliguric renal damage plasma findings cannot distinguish the condition from renal circulatory insufficiency.
6. The differentiation between the oliguria of renal circulatory insufficiency with relatively normal tubular function and of acute oliguric renal failure is best made on clinical

grounds; if a laboratory test is felt to be necessary, the urinary sodium concentration is the best indicator, but can only be interpreted if the renal blood flow was low when the specimen was secreted.

7. In most cases plasma urea or creatinine clearance and assay of one or both is adequate to diagnose and to monitor glomerular dysfunction. Assessing the concentrating capacity of the kidney may test tubular function.
8. Compared with plasma assays clearance tests are relatively imprecise and inaccurate.

RENAL FAILURE

Renal failure is defined as the condition when the kidneys are unable to maintain a normal environment. An important early feature of renal failure is high or rising serum urea and creatinine levels. Renal failure can be either of these types:

1. **ACUTE RENAL FAILURE**: This type of renal failure develops over a short period of time i.e. hours or days. The urinary output decreases to <400 ml/day. Acute renal failure may be:
   a. **Prerenal**: Sudden decrease of blood flow to the kidneys due to any cause leading to decreased glomerular filtration rate (GFR) and raised urea. This can be an acute consequence of dehydration due to severe diarrhoea, haemorrhage, shock or congestive cardiac failure.
   b. **Renal**: This occurs when blood flow to the kidney is decreased to a greater extent for certain interval of time, it leads to acute tubular necrosis (ATN), which may be irreversible. It also occurs in DIC and rejection of renal transplant.
   c. **Postrenal**: Acute obstruction to the urinary outflow:
      i) Bilateral ureteric obstruction due to stones or strictures
      ii) Bladder outlet obstruction e.g., prostatic hypertrophy
2. **CHRONIC RENAL FAILURE**: This is renal failure developing over a longer period of time i.e. months or years and is associated with both glomerular and tubular dysfunction.
3. **URAEMIC SYNDROME**: The uraemic syndrome is the terminal stage (**end stage**) of the kidney disease. It comprises symptoms, physical signs and abnormal laboratory findings that result from the failure of the kidney to maintain adequate excretory, regulatory as well as endocrine functions.

LABORATORY INVESTIGATIONS IN RENAL DISEASE

Renal disease is diagnosed and evaluated by following investigations:

1. **Serum Urea**: This is the most commonly carried out test for the assessment of kidney disease. But it should be kept in mind that serum urea can be as high as 20 mmol/L in the absence of intrinsic renal disease. It is because extra-renal factors e.g., protein diet and dehydration can cause increased urea level.
2. **Serum Creatinine**: This is a better indicator of the decreased renal function. However, slight increase in creatinine value can occur due to extra-renal factors e.g., intake of roasted meats or medicines like cephalosporins.
3. **Creatinine Clearance**: Creatinine clearance is a very sensitive method of diagnosing renal failure at its early stage. A reduced creatinine clearance is an indicator of decreased GFR and shows substantial glomerular damage. Serum urea and creatinine increase only when the creatinine clearance falls to less than 50%.
4. **Serum Sodium**: Serum sodium is usually normal in most of the renal diseases however; it may be decreased in both acute and chronic renal disease.
5. **Fractional Excretion of Sodium**: The fractional excretion of sodium (FENa%) represents the fraction of filtered sodium that is ultimately excreted in the urine. It is a very useful index to differentiate between prerenal failure from ATN and nephrotoxic renal failure. Early diagnosis of the two types of acute renal failures is clinically very important because treatment modalities are entirely different in these two disease entities. For the calculation of FENa (%) determinations of serum and urinary concentrations of sodium and creatinine are required:

$$\text{FENa}(\%) = \frac{\text{Urinary Na}}{\text{Serum Na}} \times \frac{\text{Serum Creatinine}}{\text{Urinary Creatinine}} \times 100$$

   In prerenal uraemia this ratio is always less than 1% while it is greater than 1.5% in ATN.
6. **Serum Potassium**: Hyperkalaemia is an important finding in acute renal failure and in the uraemic syndrome.
7. **Plasma and Urine Osmolality**: Plasma and

urine osmolality measurements are valuable in assessing the homeostatic function of the kidney. The normal plasma osmolality is 285±10 mmol/Kg while urine osmolality depends on the state of hydration of the body. It ranges from 50 to 1200 mmol/Kg. Determination of osmolality is useful only when plasma and urine osmolality are measured at the same time.

a. Measured Osmolality: Osmolality is measured with the help of an instrument named osmometer, which works on the principle of freezing point depression. It is a measure of all the osmotically active particles. The measured osmolality of serum (or plasma) is 285 ± 10 mmol/kg.
b. Calculated Osmolality: Rough estimate of osmolality can be obtained with the help of following formula:

$$\text{Plasma osmolarity} = (1.86 \text{x} \text{Sodium}) + \text{Urea} + \text{Glucose} + \text{Potassium}$$

c. Osmolar Gap: Osmolarity gap is difference between the measured and calculated osmolality. It is normally less than 10 mmol/L. In the presence of large amount of unmeasured substances like ethanol this gap is increased.

UREA ESTIMATION

Urea can be analysed by two types of methods:

Direct Methods: The only direct method is diacetyl monoxime method. In this method urea is heated with diacetyl monoxime in acid solution and reaction is catalysed by the addition of thiosemicarbzide and ferric ions, a yellow colour develops the intensity of which is proportional to the concentration of urea in the sample. This method is obsolete and is not in use now.

Indirect Methods: In these methods urea is first hydrolysed to ammonia by urease followed by estimation of ammonium ions:

$$\text{Urea} + H_2O \xrightarrow{\text{urease}} 2\,NH_4 + CO_2$$

Commonly used methods based on urease reaction are:

- Coupled-Enzyme Assay
- Urease Berthelot method
- Urease Nesselerisation method

Principle:

Coupled Enzyme Assay: Ammonium ions liberated in the urease reaction is reacted with another enzyme Glutamate Dehydrogenase (Glu DH)

$$2\text{-Oxoglutarate} + NH_4 + NADPH \xrightarrow{\text{GluDH}} NADP^+ + H_2O + \text{Glutamate}$$

Decrease in absorbance at 340 nm due to conversion of NADPH to $NADP^+$ is measured.

Urease Berthelot method: The ammonia formed during urease action on urea, reacts with phenol in the presence of hypochlorite to form an indophenol, which gives a blue coloured compound in alkaline *p*H with nitroprusside acting as a catalyst.

Urease Nesslerisation method: Ammonium ions liberated after the urease action, in the presence of Potassium Iodide and Mercuric Iodide (Nessler`s reagent) is changed into a yellow coloured iodide compound, which is measured calorimetrically. This method is obsolete and is not in use now.

Procedure: Follow the detailed procedure as per standard instruction provided by the manufacturer of the commercial kit in use.

Reference range:
Serum: 1.7 - 8.3 mmol/L
Urine: 333 - 583 mmol/24 h
Conversion factor: See Table 2.6 on page 10.

CREATININE ESTIMATION

Principle: Jaffe`s reaction described in 1886 is still the basis of the methods used for creatinine estimation. The reaction takes place between creatinine and picrate ions formed in alkaline medium:

$$\text{Creatinine} + \text{Picric acid} \xrightarrow{\text{Alkali}} \text{Creatinine - picrate complex}$$

This Creatinine-picrate complex is a red-orange product and it absorbs light at 510 nm. Two types of methods of creatinine estimation based on this reaction are widely used:

1. **Endpoint Assay**: Traditionally Jaffe`s method of creatinine estimation was developed as an endpoint assay allowing 10-15 min for colour development. The assay was non-specific due to many substances other than creatinine producing similar colour. It used to be done with or without deproteinisation.
2. **Kinetic Assays**: Kinetic Jeffe`s methods are based on the fact that the rate of colour formation is proportional to the concentration of creatinine in the sample. Kinetic procedures of creatinine estimation have now become very popular because of the following advantages:
   a. **Simplicity**: These assays are simple to perform as compare to the classical end point methods. Because of the smaller volume of the sample, deproteinisation can be dispensed with.
   b. **Rapidity**: The tests can be performed within two min.
   c. **Avoidance of interference**: Specificity of test markedly improves. Both positive and negative interferences are avoided by monitoring the test in critical time

window of 20-80 seconds because the colour developed in this period is predominantly due to creatinine-picrate complex.

Reagents

1. Picric acid: Make saturated solution of picric acid (see footnote on page 83).
2. Sodium hydroxide, 0.75 mol/L: Dissolve 30 g NaOH in water to make 1L. Store in a tightly stoppered, polythene bottle.
3. Working solution: Make before use by mixing equal volumes of picric acid and sodium hydroxide solutions.

Procedure: Depending upon method, follow the detailed procedure as per standard instructions provided by the manufacturer of the commercial kit in use. For reagents prepared as above follow the procedure below:

Kinetic measurement

Mark two tubes as test and blank. Add 100 µl sample and water respectively to 1 ml working regent. Measure absorbance of both at 60 and 120 seconds at 492 nm. Calculate ΔA by subtracting A^1 from A^2 of blank from sample. Read result form calibration curve or calculate by the factor. Modern semiautomated instruments can be programmed to give direct result.

Reference range

1. Serum / Plasma:
   a. Male: 62-115 µmol/L
   b. Female: 53-97 µmol/L
2. Urine:
   a. Male: 8.6-16.1 mmol/L
   b. Female: 6.7-12.3 mmol/L

Conversion factor: See Table 2.6 on page 10.

CREATININE CLEARANCE

Creatinine clearance is defined as the amount of plasma (ml) cleared of creatinine in unit time (per min). It is an assessment of glomerular filtration rate (GFR) and thus the glomerular function.

Procedure:

1. Patient is instructed to collect 24-hour urine sample. He she should be clearly explained the procedure of 24 h urine collection. Urine volume is measured (see URINE EXAMINATION Timed specimen on page 77) in ml and noted on patient's request form.
2. Blood sample should be collected at any time during these 24 hours.
3. Creatinine estimation on both serum and urine samples is performed by one of the methods described above.
4. Creatinine in urine is usually very high and is measured in mmol/L whereas serum creatinine is measured in µmol/L. Before starting calculations both should be brought to µmol/L by multiplying urinary creatinine by 1000.
5. Creatinine clearance is calculated with following formula:

$$\text{Creatinine Clearance} = \frac{\text{Urine creatinine} \times \text{Urine volume in ml}}{\text{Serum creatinine} \times \text{Time in min}}$$

Reference range:

Male: 90-135 ml/min
Female; 80-125 ml/min

Interpretation

Precision and validity: Following factors make it less precise and accurate than plasma urea or creatinine levels:

1. Urine and plasma creatinine are assayed. The combined imprecision of two assays is more than the one.
2. Timed urine collection and measurement of volume is a source of large error as the accurate collection of urine is difficult.
3. Both creatinine and urea may be partly destroyed by bacterial action in infected or old urine. This error increases with increasing length of the collection period. The measurement has now largely been replaced by either serum urea and/or creatinine or by radioisotope renal studies ($Tc^{99}m$ DMSA static renal scan, $Tc^{99}m$ DTPA dynamic renal study with Furusemide washout test & GFR estimation, Vesico-ureteric reflux study, Bladder residual urine volume estimation, Renal transplant scintigraphy) (page 418). However, reduction in creatinine clearance indicates reduced glomerular filtration. This occurs in renal disease involving damage to substantial number of glomeruli.

46. ELECTROLYTES AND ACID BASE EVALUATION

PATHOPHYSIOLOGY

1. Homeostatic mechanisms for sodium and water are interlinked. Potassium and hydrogen ions often take part in exchange mechanisms with sodium.
2. Aldosterone secretion is the most important factor affecting body sodium content.
3. Aldosterone secretion is controlled by the rennin-angiotensin mechanism, which responds to changes in renal blood flow.
4. Antidiuretic hormone (ADH) secretion is the most important factor affecting body water excretion.
5. ADH secretion is controlled by changes in plasma osmolality, which normally depends on the plasma sodium concentration. A contraction in plasma volume in pathological states may also stimulate its secretion.
6. Distribution of fluid between intra- and extracellular fluid compartments depends on the osmotic difference across the cell membrane. Changes in gradient are usually due to changes in extracellular sodium concentrations.
7. Clinical effects of disturbances of water and sodium metabolism are due to:
   a. Changes in extracellular osmolality, dependent mainly on sodium concentration. In pathological states plasma urea and glucose concentrations and ingested solutes can be important;
   b. Changes in circulating volume.

SODIUM AND POTASSIUM ESTIMATION

Following methods are in use for sodium and potassium estimation:

- Flame photometry
- Ion selective electrode (ISE)
- Colorimetric determination

Specimen:
Serum, plasma or whole blood can be used for electrolyte determination. Plasma sample obtained with heparin as anticoagulant and separated within min by centrifugation is the ideal one. Care should be taken, however, that lithium or ammonium salts of heparin are used instead of sodium or potassium salts. Spot urine samples can be used but a 24-h sample has the advantage because of availability of reference intervals.

FLAME PHOTOMETRY

For details about the instrument, principle and procedure see section on FLAME PHOTOMETER on page 18.
Calibrators: Commercial calibrators are convenient and are widely used. Three levels are available: high, medium and low according to the concentration of sodium and potassium.
Dilutions: The dilution ratio for calibrators, samples and controls is selected according to the instrument response at the time of installation of the instrument. Dilution can be carried out with an auto-dilutor provided with the instrument.

ION SELECTIVE ELECTRODE (ISE)

Principle: An Ion-Selective Electrode (ISE) produces a potential that is proportional to the concentration of an analyte. Making measurements with an ISE is, therefore, a form of potentiometry. The most common ISE is the *p*H electrode, containing a thin glass membrane responding to the H^+ concentration in a solution.
Instrumentation: ISEs consist of the ion-selective membrane, an internal reference electrode, an external reference electrode, and a voltmeter. An ISE is shown on page 24. The available instruments usually have sodium, potassium and lithium (or chloride, calcium and bicarbonate) electrodes in various combinations. The procedure is a simple one. The instrument aspirates undiluted sample and direct result is displayed on screen or is printed by the built-in printer. The instruments are self-calibrating. ISEs are very expensive and delicate structures. These have to be kept in buffer all the time. Drying will destroy the delicate measuring membrane and render the ISE as useless.

COLORIMETRIC METHOD

Various colorimetric methods have been developed. These include:

- Enzyme activation methods
- Photometry using magnesium urinyl acetate
- Turbidimetric method using tetraphenylboron
- Macrocyclic chromophores

These colorimetric assays for sodium and

potassium can be employed as back up for Flame photometry or ISE.

Preparation of Standard Solutions: Combined sodium/potassium standards in combination with or without lithium are in use: A combined standard of 140/5/1 mmol/L can be prepared by dissolving 8.19 g dried, analytical grade sodium chloride 0.3725 g analytical grade potassium chloride and 0.068 g lithium sulphate in water making the volume to one litre. Commercially prepared standards are now available.

Interference: Lipaemic specimens cause interference in the results of sodium and potassium. Haemolysis interferes with the results of potassium levels.

Reference Ranges

Sodium:

Serum: 136-145 mmol/L

Potassium:

Serum: 3.5-5.1 mmol/L

Clinical significance

1. Pathological increase of sodium (hypernatraemia)
   a. Severe dehydration
   b. *Hyperadrenalism (Cushing's syndrome*) in which excessive reabsorption of sodium in renal tubules occurs as a result of overproduction of adrenal corticosteroids.
   c. *Comatose diabetics* having treatment with insulin as some Na in cells is replaced by K.
   d. *Nasogastric feeding* of patients with solution containing a high concentration of proteins, without sufficient fluid intake.
   e. *Diabetes insipidus* (deficiency of antidiuretic hormone) without sufficient intake of water to cover the fluid loss.
2. Pathological decrease of sodium (hyponatraemia)
   a. A large loss of gastrointestinal secretions occurring with:
      i) Diarrhoea
      ii) Intestinal fistulae
      iii) Severe GI disturbances
   b. Hypernatraemia occurs when replacement is made with water only.
   c. The acidosis of diabetes mellitus before the coma stage, when large amount of Na and K are excreted into the urine as salts of the ketoacids, with replacement of water because of thirst.
   d. Renal disease with malfunction of the tubular ion exchange of Na^+ for H^+ and K^+ (salt losing nephritis).
   e. Addison's disease, with depressed secretion of aldosterone and corticosteroids.
   f. Diabetes insipidus (posterior pituitary deficiency) with compensatory intake of water
3. Pathological decrease of potassium (hypokalaemia)
   a. Due to loss of potassium from the body:
      i) Prolonged vomiting
      ii) Diarrhoea
      iii) Loss through intestinal fistulae
      iv) Secondary hyperaldosteronism
      v) Cushing's syndrome and steroid therapy
      vi) Primary hyperaldosteronism
      vii) Carbenoxolone therapy
      viii) Renal tubular acidosis
      ix) Renal tubular failure
      x) Fanconi syndrome
   b. Due to reduced potassium intake e.g., chronic starvation
   c. Due to redistribution in the body
      i) Glucose and insulin therapy
      ii) Familial periodic paralysis
      iii) Alkalosis
   d. Certain carcinomas that secrete ACTH cause lowering of K by adrenal cortex stimulation to produce excessive amounts of steroids.
4. Pathological increase of potassium (hyperkalaemia)
   a. Gain of potassium to the body
      i) Overenthusiastic potassium therapy
      ii) Failure to stop therapy after correction
   b. Failure of renal secretion
      i) Hypoaldosteronism
      ii) Diuretic working on distal tubules; spironolactone, amiloride and triamterine
      iii) Renal glomerular failure
   c. Redistribution in body
      i) Severe tissue damage
      ii) Acidosis
      iii) Hypoxia
      iv) Diabetic ketoacidosis
      v) Shock

CHLORIDE ESTIMATION BY TITRATION

Principle

Chlorides are titrated with mercuric nitrate in an acidic medium to form mercuric chloride. This mercuric chloride is in the un-dissociated form and so does not react with the indicator. At the end of titration when an excess of mercuric ions is added, they form a complex with diphenyl carbazone indicator giving a violet blue colour to

the solution.

$Hg(NO_3)_2 + 2NaCl \rightarrow HgCl_2$(un-dissociated) $+ 2NaNO_3$

Excess Hg ions + diphenyl carbazone $\rightarrow$ violet blue coloured complex

Specimen: Serum, plasma, spinal fluid, non-diluted urine and any other biological liquid can be tested. Protein free filtrate give better endpoint violet blue colour on adding the first drop of excess mercuric nitrate solution. Two ml of Folin-Wu nitrate (page 50) may be taken for titration with mercuric nitrate.

Reference Range:

Serum: 98-108 mmol/L

Urine: 110-250 mmol/24 h

Conversion factor: See Table 2.6 on page 10.

Sources of errors: Some drugs have physiologic effect and others have chemical interference.

Drugs causing physiologic effects are, Acetazolamide, Chloride, Oxyphenbutazone, Phenylbutazone, ACTH, Corticosteroids, Ethacrynic acid, Mercurial diuretics, Furusemide, Triamterene.

Interpretation:

1. Pathological Increase:
   a. Dehydration
   b. Certain types of renal tubular acidosis
   c. A patient with primary CO_2 deficit (respiratory alkalosis) caused by drugs or states (hysteria, anxiety, fever) that stimulates the respiratory centre and cause over-breathing.
2. Pathological Decrease:
   a. Metabolic acidosis (high anion gap)
   b. Uncontrolled diabetes.
   c. In renal disease (phosphate ion retention accompanies impaired glomerular filtration).
   d. Pyloric stenosis
   e. Intestinal obstruction with prolonged vomiting
   f. Salt losing nephritis
   g. Metabolic alkalosis

CALCIUM ESTIMATION

Calcium is the most abundant cation in the body and amounts to 25 to 35 mol (1.0-1.4 kg) in the adult. Over 99% is in the bones and teeth. The small part of the body calcium present in plasma and other extracellular fluids is vital. It maintains the conditions for neuromuscular transmission, glandular secretion, activity of enzyme systems and blood coagulation. The total plasma calcium is composed of a **protein bound non-diffusible** fraction and a **diffusible part** most of which is functionally important **ionised** calcium with a small amount present in non-ionic form. Three hormones are involved in the regulation of plasma level of calcium. They are **parathormone**, **calcitonin** and Vitamin D (1, 25-dihydroxycholecalcifcrol). Calcium is mainly absorbed from jejunum under the influence of vitamin D.

Principle: The determination of total calcium in biological fluids is based on the formation of a blue complex when calcium reacts with methylthymol blue in an alkaline medium.

Specimen: Serum is the preferred specimen for the measurement of total calcium. Heparinised plasma can also be used. Citrate, oxalate and EDTA anticoagulants should never be used because they interfere by forming complexes with the calcium.

Urine Sampling: Collect 24 h urine in calcium free container. Place 10 ml concentrated nitric acid in the container to avoid phosphate precipitation. Make 1/10 dilution of urine before estimation.

Procedure: Follow the detailed procedure as per standard instructions provided by the manufacturer of the commercial kit in use

Interference: Haemolysis, icterus, lipaemic, paraproteins and magnesium interfere with the results of total calcium. Lipaemic specimens should be clarified by high-speed centrifugation. *p*H changes interfere with the results of ionised calcium.

Reference Range

Serum: 2.25 -2.75 mmol/L

Urine: 1.25-7.0 mmol/day

Conversion factor: See Table 2.6 on page 10.

Corrected total serum calcium: Total calcium level shows variation due to changes in serum proteins. A formula is devised to give a corrected total calcium value.

Corrected total calcium (mmol/L)=Total Ca+0.02(40–serum albumin)

IONISED CALCIUM ESTIMATION

Measurement of ionised calcium is preferred because it is the clinically important fraction. Ionised calcium is measured by ion selective electrode (ISE) with or without sodium, potassium, chloride or bicarbonate. For details see ION SELECTIVE ELECTRODE (ISE) on page 335.

Reference range: 0.16 to 1.32 mmol/L

Interpretation:

Hypercalcaemia

- Primary hyperparathyroidism
- Hyperthyroidism
- Chronic acidosis
- Primary and secondary malignant disease of bone (Osteolytic)
- Myelomatosis
- Immobilisation

Overdose of Vitamin D
Hypersensitivity to Vitamin D
Sarcoidosis
Excess dietary intake of alkali with calcium

Hypocalcaemia

Hypoparathyroidism e.g., thyroidectomy, irradiation, iron overload
Chronic renal failure
Decreased calcium intake, decreased calcium in diet, malnutrition
Decreased absorption e.g., Malabsorption syndrome, surgical resection of gut
Chronic alcoholism
Childhood malnutrition
Lactation
Malabsorption
Acute pancreatitis
Hypoparathyroidism
Digitalis intoxication
Prolonged intravenous feeding
Renal causes:
Chronic glomerulonephritis
Aldosteronism
Renal tubular reabsorption defects

Hypermagnesaemia is seen in following conditions:

Dehydration
Severe diabetic acidosis
Addison's disease
Uraemia

MAGNESIUM ESTIMATION

Magnesium is a trace element of the body. The adult body contains about 24 g of magnesium most of which is present in bone. Together with potassium, magnesium is a major intracellular cation. Magnesium ions are essential for maintenance of the functional and structural integrity of the myocardium. It is an essential factor in many important enzymatic reactions.

Specimen: Blood sample for serum magnesium estimation should be obtained without venous stasis. As magnesium concentration in erythrocytes is much greater than in serum, the specimen should be separated from erythrocytes as soon as possible and haemolysis should be avoided.

Procedure:

Colorimetric method: Many colorimetric methods are in use for estimation of serum magnesium. Calmagite colorimetric method is more useful for both manual and automated use. The method is based on the principle that calmagite combines with magnesium to form a coloured complex which is measured at 545. The method is simple and rapid and results are reliable. Commercial kits based on this method are available.

Atomic absorption spectrophotometry: Atomic absorption spectrophotometry (pagee 18) is the preferred technique for the estimation of magnesium in biological specimens. The method is quick and accurate once the specimen is prepared properly. The sample preparation requires release of magnesium from proteins by treatment with HCl or trichloracetic acid followed by centrifugation. The supernatant is then analysed by atomic absorption spectrophotometry.

Interference: Icterus and lipaemia interfere the results. Lipaemic specimens should be clarified by high-speed centrifugation.

Reference range: Serum: 0.6-1.1 mmol/L

Clinical significance:

Hypomagnesaemia is usually seen in the following conditions:

PHOSPHATE ESTIMATION

The body contains about 530 g phosphorus and most of it is present in the bones. Phosphorus also forms a part of many substances like some proteins, lipids and nucleic acids. It plays a role in acid base regulation. The phosphorus in the blood is present as inorganic phosphorus and organic or ester phosphorus (phospho-lipoprotein).

Principle: Inorganic phosphate reacts with molybdic acid forming a phosphomolybdic complex. Its subsequent reduction in alkaline medium causes a blue molybdenum colour formation, the intensity of which is proportional to the amount of phosphorus present.

Specimen: Serum or heparinised plasma is preferred specimen for estimation. Anti coagulants such as citrate, oxalate and EDTA should not be used because they interfere by forming complexes with phosphorus.

For phosphorus determination in urine, collect a 24 h specimen into a bottle containing 10 ml of 10% HCl to avoid phosphorus precipitation. Mix, dilute the sample 1:10 with distilled water for use in test procedure (0.5 ml).

Procedure: The details of reagents and procedure are provided with the reagent kit available from different companies.

Interference: Dirty glassware, haemolysed, icteric and lipaemic specimens interfere the results.

Reference Range

Serum: 0.80-1.65 mmol/L
Urine: 9.6-32.3 mmol/day

Clinical Significance

Increased serum levels are found in chronic nephritis rising progressively with increasing renal failure. There is a moderate increase in

hypothyroidism. Decrease phosphate level is seen in rickets, osteomalacia, in primary and secondary hyperparathyroidism.

pH AND BLOOD GAS ANALYSIS

HYDROGEN ION HOMEOSTASIS

1. CO_2 is of central importance in hydrogen ion homeostasis. Arterial blood PCO_2 is controlled by the respiratory centre at about 5.3 kPa (40 mmHg).
2. At a PCO_2 of 5.3 kPa the carbonate deydratase mechanism in erythrocytes and renal tubular cells maintains the plasma [HCO_3^-] at about 25 mmol/L.
3. The H^+ produced in erythrocytes is buffered by haemoglobin. This mechanism is of physiological importance, but, because of its limited capacity, only plays a minor role in correcting abnormalities in H^+ balance.
4. Renal tubular cells secrete H^+ into the urine in exchange for Na^+. Hydrogen ion secretion is essential for HCO_3^- 'reabsorption' and net generation.
5. Normal urine is almost HCO_3^- free. Generation of HCO_3^- to replace its use in buffering depends on the availability of urinary buffers especially HPO_4^{--}.
6. Renal correction of either acidosis or alkalosis depends on a normal GFR.
7. A reduction in the ratio [HCO_3^-]:PCO_2 causes acidosis. Although the ratio is normal in compensated acidosis, both [HCO_3^- and PCO_2 are abnormal.
8. An increase in the ratio [HCO_3^-]:PCO_2 causes an alkalosis. In compensated alkalosis the ratio is normal but the levels of both HCO_3^- and CO_2 are abnormal.
9. Oxygen is transported in blood bound to haemoglobin.
10. Factors that affect the affinity of haemoglobin for oxygen include the *p*H of the blood and the erythrocyte concentration of 2,3-diphosphoglycerate (DPG)

Instrument

Blood gas analyser having ISEs for pH, pCO_2 and pO_2 measures these parameters. The Rest of the parameters are calculated. The sample consisting of heparinised blood in an airtight syringe or capillary tube is aspirated through the fluidics of analyser, which contains these electrodes. The modern instruments are self-calibrating and are temperature controlled. For details see ION SELECTIVE ELECTRODE (ISE) on page 335.

Reagents

1. Calibrations buffers
   a. *p*H 7.382±0.005 at 37°C
   b. *p*H 6.838±0.005 at 37°C
2. Flush solution concentrate
3. PCO_2 electrolyte fill solution
4. PO_2 electrolyte fill solution

Specimen: Arterial samples are collected in a heparinised syringe and immediately sent to laboratory under ice-cold water or in ice bag. Test should be done within 30 min of taking the sample to minimise changes in the blood gases.

Procedure: The user is advised to follow the instruction manual provided with the apparatus.

Reference Range (Arterial blood at 37°C)

*p*H	7.35-7.45
PCO_2	4.5-6.0 kPa
PO_2	11-15 kPa
HCO_3^-	23-30 mmol/L
O_2 saturation	95%

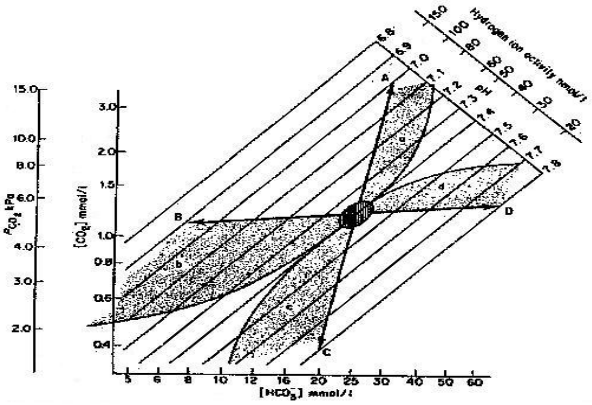


Figure 46.1: Nomogram for interpretation of acid basc disorders.

Interpretation

1. <u>Low PO_2 and normal or low PCO_2</u>: Carbon dioxide is much more soluble in water than oxygen. Arterial PCO_2 is therefore, less affected than PO_2 in pulmonary oedema; it may even be low because of respiratory stimulation (Figure 46.1).
2. Arterial blood is 95% saturated with oxygen. Increased respiration cannot increase oxygen carriage from normal alveoli, but can reduce the PCO_2. If some alveoli have a normal blood supply, but are poorly ventilated (ventilation-perfusion mismatch) the mixture of 'shunted' blood with that from normal alveoli results in a low PO_2 and a normal or low PCO_2 in systemic arterial blood.
3. The PO_2 is low and the PCO2 is high if there is widespread alveolar hypoventilation, because neither gas can be exchanged

adequately.

LACTIC ACID ESTIMATION

Blood Lactate is mainly derived from pyruvate when there is hypoxia, which prevents further metabolism of pyruvate metabolised in citric acid cycle. When the lactic acid level rises above 7 mmol/L, the condition is called lactic acidosis. Tissue hypoxia due to the poor tissue perfusion of the 'shock' syndrome is the commonest cause of lactic acidosis. Moderate increases occur in muscular exercise, severe anaemia, acute asthmatic attack, convulsions or diabetic coma. Greater changes are seen in shock with peripheral circulatory failure. Other causes are severe illness, biguanides and von Gierke's disease.

Specimen: The blood should be collected with minimal venous stasis after resting the patient for 1-2 h. Prevent glycolysis by addition of fluoride as preservative.

Principle (Enzymatic method): A photometric method using enzyme is more specific and simple. Lactate is converted into pyruvate in the presence of lactate dehydrogenase, with the simultaneous conversion of NAD^+ into NADH under alkaline conditions. The amount of NADH formed is measured at 340 nm.

Reagents and procedure: Follow the detailed procedure as per standard instructions provided by the manufacturer of the commercial kit in use.

Reference range: Plasma; 0.75-2.0 mmol/L

47. PURINE AND URATE METABOLISM

In man uric acid is the major end product of purine metabolism. The bulk of uric acid excreted in urine comes from degradation of endogenous nucleic acids. Hyperuricaemia is the term applied when serum uric acid concentration rises above 415 µmol/L (7.2 mg/dl) in men or 357 µmol/L (6 mg/dl) in women. Hyperuricaemia occurs when either there is increased rate of nucleic acid turnover (malignancy, tissue damage, starvation), increased rate of synthesis of purines (primary gout) or there is decreased rate of renal excretion of urate (glomerular dysfunction, thiazide diuretics, acidosis). Primary hyperuricaemia and gout have a familial incidence. Both are rare in women of child-bearing age. Gout occurs when monosodium urate precipitates in the tissues. These deposits of urates are responsible for the clinical signs and symptoms (Figure 47.1). About 10% of gouty patients develop urate stones. Urate crystals when seen under polarising light are needle like in shape and exhibit strong negative birefringence (Figure 9.18 on page 88). Hyperuricaemia is rare and usually unimportant. It occurs in the very rare inborn error, xanthinuria.

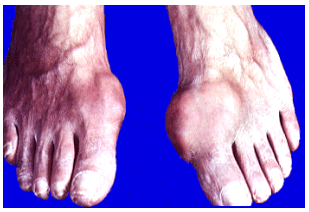


Figure 47.1: Gout

URIC ACID ESTIMATION

PHOSPHOTUNGSTIC ACID METHOD

Principle: Proteins in serum are precipitated with tungstic acid. Uric acid in the supernatant reduces the phosphotungstic acid into tungsten blue in an alkaline medium of sodium bicarbonate. The colour of the tungsten blue is proportional to the uric acid concentration and is read at 660 nm.

Specimen: Use fresh serum, separate clot from the blood without any delay. The serum is stable for three days at 25°C and 3-7 days at 4°C.

Reagents and procedure: Follow the detailed procedure as per standard instructions provided by the manufacturer of the commercial kit in use.

Precautions:

1. The *p*H for precipitation of the proteins should be below 3 in order to avoid loss of uric acid.
2. The precipitated proteins should be removed by centrifugation rather than by filtration in order to avoid loss of uric acid by adsorption to the filter paper.
3. The *p*H must be alkaline.

Disadvantage: This method is subjected to interferences from glucose, ascorbic acid, glutathione, acetyl salicylic acid, caffeine and haemolysis.

URICASE METHOD

Principle: Uric acid is oxidised in the presence of enzyme uricase to allantoin and hydrogen peroxide (H202). The hydrogen peroxide can be measured by means of catalase peroxidase linked reactions. In these reactions ethanol is catalysed by catalase and produces acetaldehyde. The acetaldehyde is further oxidised to acetate by aldehyde dehydrogenase in the presence of NAD, which is reduced to NADH. The increase in absorbance at 340 nm is measured for sample and standard and then concentration of sample is calculated. In another method the hydrogen peroxide is detected by a chromogenic oxygen acceptor dichlorophenol in the presence of peroxidase. The red colour is measured photometrically.

Advantages: This method is specific for uric acid and its linearity is up to 4 times the upper reference range.

Reference range:

Male: 150-415 µmol/L

Female: 90-357 µmol/L

Conversion factor: See Table 2.6 on page 10.

Interpretation:

1. PATHOLOGICAL INCREASE
   a. Gout, Idiopathic hyperuricaemia, renal disease, Toxaemia of pregnancy
   b. Leukaemias/lymphomas/Myeloproliferative disorders
   c. Resolving pneumonia, Psoriasis
2. PATHOLOGICAL DECREASE
   a. ACTH or Corticosteroid administration
   b. Drugs e.g., probenecid, aspirin and pencillamine.
   c. Drugs blocking uric acid synthesis e.g., allopurinol
3. PHYSIOLOGIC VARIATIONS: Stress, Alcohol

48. IRON METABOLISM

Iron is an essential trace element. In the body nearly all of it is linked with proteins; those containing **haem** i.e., haemoglobin, myoglobin, cytochromes and those not containing haem i.e., ferritin, transferrin, flavoproteins and oxygenases. An average adult male has about 4 g of body iron, while females have 3 g due to less reserve and relatively low haemoglobin concentration. About 70% of total iron is found in haemoglobin and myoglobin, remaining 30% is present in storage pool. Iron stores are mainly in the reticuloendothelial system of liver, bone marrow and spleen in the form of protein complexes as **ferritin** and ferritin aggregates (**haemosiderin**). Iron is transported by a globulin called **transferrin** (Figure 48.1). Its concentration may be measured directly, or indirectly as the total iron binding capacity (**TIBC**). It rises in iron deficiency and falls in iron overload. There is no physiological control mechanism for iron excretion and body stores are determined by control of absorption. There is a daily loss of about 1 mg iron because of the normal shedding of mucosal and epithelial cells, and loss of erythrocytes in urine and faeces. Therefore, iron intake of about 1 mg per day is sufficient for men and 1.5-2.0 mg for women. Lactating and pregnant women require about 3 mg. Iron is absorbed in the duodenum and upper small intestine (slow release preparations are useless). Haem iron (derived from dietary haemoglobin and myoglobin) is more efficiently absorbed than iron from non-haem sources. Factors influencing iron absorption include:

1. Dietary haem and non-haem moieties
2. Gastric secretions and hydrochloric acid reduce ferric (Fe^{+++}) iron to the absorbable ferrous (Fe^{++}) form.
3. Ascorbic acid, sugars and amino acids form soluble iron compounds enhance the absorption.
4. Phosphates (milk), oxalates and phytates (in vegetables) and tannates (in tea) form insoluble compounds with iron and inhibit the absorption.
5. Increased intestinal motility reduces iron absorption.
6. Anaemia even if not due to Iron deficiency, increases the absorption of iron.
7. Increased erythropoiesis increases uptake of iron.

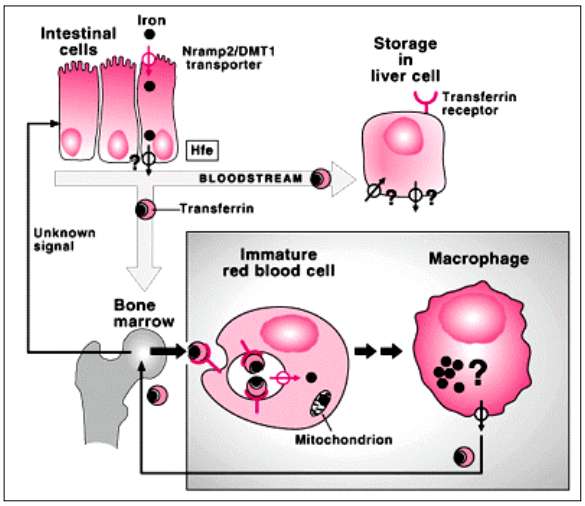


Figure 48.1: Metabolic pathways of iron metabolism.

SERUM IRON

Serum iron measurement is of little value in the investigation of iron metabolism, except in relation to haemochromatosis and in the diagnosis and management of iron poisoning and overload. A fall in serum iron concentration is a late feature of iron deficiency. Isolated, serum iron levels have limited diagnostic value. This should always be interpreted with total iron binding capacity (TIBC) and transferrin saturation because a variety of physiological and pathological factors influence these levels.

Physiological variations

1. **Sex**: 10-20% higher in males.
2. **Circadian Rhythm**: Highest in the morning and lowest in the evening. Fluctuations can occur up to 50%.
3. **Day to day**: Variations in serum iron may be two to three folds.
4. **Menstrual cycle**: Very low values may be found immediately prior to, and during menstruation.
5. **Pregnancy**: Increased due to increased transferrin synthesis. The increased demand for iron may overshadow the effect of increased transferrin and may be reflected by a low plasma iron.
6. **Oral Contraceptives**: Increased levels are due to protein synthesis by oestrogen.

SERUM IRON DETERMINATION

Principle: Iron is released from transferrin iron complex by acid *p*H and is reduced to ferrous form (Fe^{++}) by a reducing agent. The reduced form of iron reacts with chromogen. The intensity of colour is proportional to iron concentration. Widely used chromogens are bathophenanthroline and ferrozine.

Reagents and procedure: Follow the detailed procedure as per standard instructions provided by the manufacturer of the commercial kit in use.

Reference Range

Male: 9-29 µmol/L (50-160 µg/dl)
Female: 7-27 µmol/L (40-150 µg/dl)

Conversion Factor: See Table 2.6 on page 10.

Interpretation

1. **Pathological increase**
   a. Increased red blood cells destruction (**haemolytic** anaemia).
   b. **Ineffective** or decreased red cell formation (pernicious anaemia, aplastic anaemia).
   c. **Blockage in haem synthesis** (lead poisoning, pyridoxine deficiency).
   d. **Increased release** of storage iron (acute hepatic necrosis).
   e. **Increased intake** or impaired control of iron absorption (Ingestion of large amount of iron, haemochromatosis, haemosiderosis).
   f. **Multiple transfusions** (thalassaemia).
2. **Pathological decrease**:
   a. **Low dietary intake** causes deficiency in the body and leads to microcytic hypochromic anaemia.
   b. **Loss of iron** or **increased demand** (acute and chronic blood loss, late pregnancy).
   c. **Impaired release** of stored iron from reticuloendothelial cells (infection, neoplasia, rheumatoid arthritis).
   d. **Chronic diseases**; infection, inflammation, malignancy, connective tissue disease and renal failure are associated with blocked release of iron from stores, resulting in low serum iron in presence of normal stores.

The laboratory findings in conditions, which may affect plasma iron concentrations, are summarised in Table 48.1.

TOTAL IRON BINDING CAPACITY (TIBC)

Transferrin is a β-globulin. Usually it is only one third saturated with iron. The amount of iron that transferrin can bind to; to become fully saturated is termed unsaturated iron binding capacity and a combination or UIBC and serum iron is called total iron binding capacity (**TIBC**). It is a measure of transferrin.

Table 48.1: Biochemical findings associated with plasma iron abnormalities. N =normal; =increased level; =decreased level, ? =level variable

	Plasma concentrations			Marrow
	Iron	Transferrin	Ferritin	stores
Low iron concentration				
Before menstruation	↓	N	N	N
Iron deficiency	↓	↑	↓	↓↓
Acute illness	↓	N	N or ↑	N
Chronic illness	↓	↑	N or ↑	↑
High iron concentration				
Early pregnancy	↑	N	N	N
Late pregnancy, contraceptive	?	↑	N	N
Iron overload	↑	↓	↑	↑
Liver disease	↑	↓	↑	↑
Impaired marrow utilisation	↑	N or ↓	↑	↑
Haemolysis	↑	N or ↓	↑	↑

Principle: Known quantity of ferrous iron is added to serum in an alkaline medium to fully saturate transferrin. The excess unbound iron is removed by reacting with magnesium carbonate and estimated. By abstracting the unbound iron from the quantity originally added UIBC is determined. To this serum iron is added to get TIBC.

Reference range: Adults 45-75 µmol/L

Interpretation: The diagnostic precision may sometimes be improved by measuring both the plasma transferrin (TIBC) and iron concentration in rare situations in which doubt remains after haematological investigations. Typically, in uncomplicated iron deficiency, low plasma iron is associated with a high transferrin concentration and TIBC; that of non-iron deficiency is associated with low concentration. If iron deficiency coexists with the anaemia of chronic illness the opposing effects of the two conditions on transferrin concentration make it difficult to interpret transferrin, as well as plasma iron concentrations (Table 48.1).

FERRITIN

Circulating ferritin is in equilibrium with the stores. It is an acute phase protein, and the synthesis is increased in many inflammatory conditions. Its concentration declines very early in iron deficiency and increases in iron overload. A plasma concentration below ~10 µg/L almost certainly indicates iron deficiency. Finding a normal or low ferritin almost certainly excludes the diagnosis of iron overload. The results can be misleading if there is coexistent inflammatory disease, since accelerated synthesis may lead to normal or even high plasma concentration despite very low iron stores. High concentrations of plasma ferritin always occur in significant iron

overload, but may also be due to inflammatory conditions, malignant disease and liver diseases. The ferritin can be assayed in serum by:

1. Immunoradiometric assay (IRMA)
2. Enzyme linked immunosorbant assay (ELISA)
3. Radio immunoassay (RIA)
4. Fluorescent immunoassay (FIA)

Reference range:

Adult male: 20-300 µg/L
Adult female: 15-120 µg/L
Children: 10-140 µg/L
Newborn/infant: 25-200 µg/L

BIOCHEMICAL INVESTIGATIONS OF ANAEMIA

The clinical impression of anaemia needs to be confirmed by haemoglobin estimation, absolute values and examination of a blood film. A bone marrow examination may be needed for the diagnosis. It can be stained for iron. In rare cases in which the diagnosis is not clear, and if a bone marrow aspiration is felt to be unjustified, biochemical investigations may occasionally help. Plasma iron estimation without an assessment of transferrin concentration is uninformative (Table 48.1). An unequivocally low plasma ferritin concentration confirms iron deficiency, but a normal or high one should not be assumed to exclude it. For a diagnosis of iron overload, proof has to be obtained of increased iron stores.

THE PORPHYRIAS

Porphyrins are by-products of haem synthesis. 5-aminolaevulinate (ALA) and porphobilinogen (PBG) are precursors. The porphyrias are diseases associated with disturbed porphyrin metabolism. Most are inherited. Acute attacks, with abdominal or neurological symptoms, are a feature of the inherited hepatic porphyrias. Such attacks are potentially fatal and may be provoked by a number of drugs, the diagnosis of porphyria in the acute phase depends on the demonstration of ALA and PBG in the urine. The diagnosis of inherited porphyria must be followed by investigation of all blood relatives to detect symptomatic cases. Screening tests may be negative in some types and quantitative estimations are necessary. Both urine and faeces should be examined. Other causes of abnormal porphyrin excretion are lead poisoning, liver diseases and upper gastrointestinal bleeding. The very rare erythropoietic porphyrias cause excessive accumulation of porphyrin in erythrocytes.

INVESTIGATIONS OF SUSPECTED PORPHYRIA

Notify the laboratory and check which types of samples are required. The samples must be protected from light. A random fresh urine sample is more suitable than a 24 h sample for PBG (also see PORPHOBILINOGEN on page 84).

Suspected acute attack: Immediately test for PBG. A negative test will not exclude the diagnosis of latent porphyria.

Suspected latent porphyria: History of repeated attacks of abdominal pain or neurological symptoms may suggest acute intermittent porphyria, porphyria variegata or hereditary coproporphyria. Measure porphyrin in random sample of faeces and PBG deaminase in red cells.

Suspected porphyria with skin lesions: Blood urine and faeces should be sent for testing accordingly.

49. LIPIDS AND LIPOPROTEINS

LIPID METABOLISM

The main plasma lipids are cholesterol, triglycerides, phospholipids and free fatty acids. Of these cholesterol and triglycerides are the most commonly measured. Lipids are transported in plasma incorporated in lipoproteins. Exogenous (dietary) lipid is carried in chylomicrons. Endogenous lipid from the liver is carried incorporated in very low-density lipoprotein (VLDL), which is metabolised to low-density lipoprotein (LDL). High-density lipoprotein (HDL) is important for removal of cholesterol from cells. Enzymes modify lipoproteins and their remnants are taken up by receptors on cells, mainly in the liver. The metabolism of lipoproteins is controlled by protein components the apolipoproteins. Plasma LDL concentrations are regulated mainly by hepatic LDL receptor concentrations. The higher the plasma LDL concentration the greater the risk of ischaemic heart disease. Hyperlipidaemia may be primary or secondary to other diseases. The nature of the lipoprotein abnormality can usually be inferred from the plasma cholesterol and triglyceride concentrations. In primary hyperlipidaemia it may be necessary to define the lipoprotein abnormality more fully for the purpose of treatment. Different genetic defects may produce similar lipoprotein abnormalities. Extensive family studies are required to differentiate them. This is rarely practicable, because of the difficulty of tracing family members. Table 49.1 shows the classification of hyperlipidaemia.

Table 49.1: WHO (Fredrickson) classification of hyperlipidaemia, based on the electrophoretic pattern of the lipoproteins

Type	Electrophoretic pattern	Lipoprotein increased
I	Increased chylomicrons	Chylomicrons
IIa	Increased β-lipoproteins	LDL
IIb	Increased pre-β and β lipoproteins	VLDL and IDL
III	'Broad β' band	IDL
IV	Increased pre-β lipoprotein	VLDL
V	Increased pre-β lipoprotein and chylomicrons	VLDL and Chylomicrons

TOTAL CHOLESTEROL ESTIMATION

1. **Liebermann-Burchardt reaction**: Cholesterol is extracted by ethanol/ether, allowed to react with sulphuric acid and acetic anhydride to form green coloured molecule (Cholestahexane sulphuric acid). It is measured at 410 nm. This method is infrequently used.
2. **Abell method**: Cholesterol is extracted with zeolite, esters are chemically hydrolysed and total cholesterol is measured by Leibermann-Burchardt reaction. It is laborious and not in use.
3. **Iron-salt acid method**: A solution of $FeCl_3$ in H_2SO_4 is added to the solution of cholesterol in glacial acetic acid. Intense magenta coloured molecule (tetraenolic cation) is formed which is measured at 563 nm. Colour production is very stable. It is more sensitive as compared to other chemical methods but not frequently used.
4. **Toluene Sulfonic acid method**: *p*-TSA in the presence of H_2SO_4, glacial acetic acid and acetic anhydride reacts with cholesterol to form chromophore. The colour complex is measured at 563 nm. Bilirubin cause large positive interference. It is rarely used.
5. **Enzymatic end point method** (CHOD-PAP) method: Cholesterol esters are hydrolysed by cholesterol esterase into cholesterol and fatty acids. The cholesterol is oxidised by cholesterol oxidase into cholesternone and H_2O_2. The H_2O_2 in the presence of peroxides reacts with phenol and 4-aminophenazone to form red coloured substance (quinine). It is measured at 505 nm.

Specimen: Serum or plasma is stable at 2-8°C for 7 days.

Reagents and procedure: Follow the detailed procedure as per standard instructions provided by the manufacturer of the commercial kit in use.

Reference Range: 3.8 – 5.2 mmol/L

Conversion factor: See Table 2.6 on page 10.

HDL-CHOLESTEROL ESTIMATION

It is measured by ultracentrifugation, column chromatography, electrophoresis and enzymatic methods.

Principle: The chylomicrons, VLDL and LDL contained in the sample are precipitated by phosphotungstic acid in the presence of divalent cations (Mg^{++}). The supernatant obtained after centrifugation contains HDL-cholesterol is measured by enzymatic method.

Specimen: Serum or plasma is stable for 7 days

at room temperature.
Reagents and procedure: Follow the detailed procedure as per standard instruction provided by the manufacturer of the commercial kit in use.
Reference Range: >0.9 mmol/L

LDL-CHOLESTEROL ESTIMATION

Calculation: The establishment of a formula by Friedwald in 1972 has led to the use of a calculated LDL-Cholesterol value. The formula is based on the assumption that VLDL is only carrier and the ratio of triglycerides/cholesterol is constant (2.2/1).

$$\text{LDL - Chol} = (\text{Total Chol}) - (\text{HDL - Chol}) - (\text{TG}/2.2)$$

If triglyceride is >4.0 mmol/L then LDL-C shall be directly measured as follows:
It is measured by ultracentrifugation, column chromatography, electrophoresis, enzymatic and calculation methods.
Principle: LDL-cholesterol is precipitated. The supernatant is removed by centrifugation and precipitate is re-suspended. The LDL-cholesterol is measured by enzymatic method.
Specimen: Serum or plasma is stable for 7 days at room temperature.
Reference Range: Serum: <3.4 mmol/L

VLDL-CHOLESTEROL ESTIMATION

It is measured by ultracentrifugation, column chromatography, electrophoresis, enzymatic and calculation methods.
Calculations: $\text{VLDL - Chol} = TG / 2.2$

IDL CHOLESTEROL ESTIMATION

It is measured by ultracentrifugation, column chromatography, electrophoresis, enzymatic and calculation methods.

TRIGLYCERIDES ESTIMATION

Till 1950 triglycerides were estimated by indirect (calculation) method. Now it is obsolete and no more in practice
Colorimetric method: In 1957 Van Handle & Zilversm introduced a direct method of estimation. It is cumbersome and has four steps: Lipids extraction, Saponification, triglyceride hydrolysis, oxidation of glycerol and quantitation of formaldehyde.
Enzymatic (GPO-PAP) method: Triglycerides are hydrolysed by lipases to release glycerol and fatty acids. Glycerol is converted by glycerol kinase to glycerol phosphate. Glycerol phosphate is oxidised by glycerol phosphate oxidase to dihydroxyacetone phosphate. Hydrogen peroxide released during this reaction is exposed to phenol and 4-aminophenazone in the presence of peroxidase. A coloured compound (quinone) is formed which is measured at 505 nm.
Specimen: Serum or plasma is stable in sample for 7 days at 2-8°C.
Reagents and procedure: Follow the detailed procedure as per standard instructions provided by the manufacturer of the commercial kit in use.
Reference Range: 0.4-2.3 mmol/L

FATTY ACID ESTIMATION

Fats are extracted by ethanol-ether mixture. The extract is saponified. Fatty acids are liberated by acidification, which are finally titrated with NaOH to calculate the concentration of fatty acids. Due to technical limitations as well as limited clinical significance, fatty acid estimation is not practical anymore.

PHOSPHOLIPIDS ESTIMATION

Fats are extracted by ethanol-ether mixture. After treating the supernatant with H_2O_2, the phosphates are determined calorimetrically to derive the values of phospholipids.

LIPOPROTEINS ASSESSMENT

OVER NIGHT PLASMA APPEARANCE: Take the fresh blood sample in EDTA container and separate the plasma by centrifugation. Allow 4 ml plasma, (in 50x6 mm test tube covered with liquid paraffin) to stand overnight at 4°C. Examine the test tube next morning in bright light against dark background. If the plasma is clear, the triglyceride level is most likely normal. When the triglyceride level increases to 300 mg/dl, the plasma is usually hazy-turbid in appearance. When the plasma triglyceride level is 600 mg/dl, the plasma is usually opaque and milky. If the chylomicrons are present, a thick homogeneous creamy layer is observed at the top. An approximate relationship of these findings with lipoprotein disorders (Table 49.1) is as follows:

1. A uniformly opaque plasma -Type IV
2. An opaque plasma with a creamy layer on top -Type V
3. A thick creamy layer with generally clear plasma - Type I

ELECTROPHORESIS: The lipoproteins can be separated by electrophoresis on agarose or cellulose acetate membrane. Fractions are visualises by fat stains (Figure 49.1).

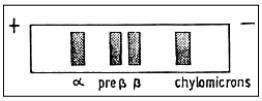


Figure 49.1: Lipoprotein electrophoresis

Following bands are seen:

- α-band for HDL
- Pre-β band for VLDL
- β-band for LDL and
- Chylomicrons at application site

ULTRACENTRIFUGATION: Lipoproteins have lower density so can be isolated from other plasma proteins by ultracentrifugation in a salt solution of specified density. The instrument is expensive and technique requires expertise.

APOLIPOPROTEINS ESTIMATION

Following are the major apolipoproteins:

1. Apo A-I (HDL, Chylomicron)
2. Apo A-II (HDL, Chylomicron)
3. Apo B-48 (Chylomicron)
4. Apo B-100 (LDL, IDL, VLDL)
5. Apo C-I (Chylomicron, HDL, VLDL)
6. Apo C-II (Chylomicron, HDL, VLDL)
7. Apo C-IH (Chylomicron, HDL, VLDL)
8. Apo E (chylomicron, HDL, VLDL)
9. Apo (a) (LDL, IDL)

These are measured by column chromatography, electrophoresis, radial immunodiffusion (RID), ELISA, EIA, FIA, RIA and immunonephalometry.

50. ROLE OF ENZYMES IN CLINICAL LABORATORY

Enzymes are proteins that act as biological catalysts, altering reaction rates and providing a means of regulating metabolic reactions. **Clinical enzymology** is the application of the science of enzymes to the diagnosis and treatment of disease. Most enzymes are present in cells at much higher concentrations than in plasma. Normal plasma enzyme level reflects the balance between rates of synthesis and release in to plasma during cell turn over, and the rate of clearance from the circulation. The enzyme activity in the plasma may be increased due to proliferation of cells, increased rate of cell turnover, cell damage, increased enzyme synthesis, or reduced clearance from plasma. Occasionally, lower than normal plasma levels occur due to reduced synthesis, congenital deficiency, or the presence of inherited variant of relatively low biological activity. Enzyme levels are therefore useful in:

1. **Assessment of cell damage and proliferation**: Plasma enzyme levels depend on the rate of release from damaged cells and the extent of cell damage. If there is no cell damage then the levels indicate the rate of cell proliferation or the degree of induction of enzyme synthesis. These factors are balanced by the rate of enzyme clearance from the circulation.
2. **Localisation of damage**: Measurement of the plasma activity of an enzyme known to be in high concentration within cells of a particular tissue may indicate an abnormality of those cells, although, a specific diagnosis may be difficult because of non-specific nature of these elevations. The diagnostic precision of plasma enzyme analysis may thus be improved by estimation of more than one enzyme, isoenzyme determination or by serial enzyme determination.

NON-SPECIFIC CAUSES OF RAISED PLASMA ENZYME ACTIVITY

Before attributing a change in plasma enzyme activity to specific disease process, it is important to exclude the presence of factitious or non-specific causes. These include peripheral circulatory insufficiency, trauma, malignancy and surgery. Artefactual increases may occur in haemolysed samples. Slight rise in plasma **Aspartate transaminase** activity occurs after moderate exercise. A large intramuscular injection may lead to a rise in plasma **creatine kinase** activity. In this situation isoenzyme determination may help in identifying skeletal muscle as the tissue of origin. Some drugs, such as the anticonvulsants (phenytoin and phenobarbitone) may induce synthesis of microsomal enzymes such as γ-glutamyl transferase, and thus increase its plasma activity in the absence of disease. Enzyme activity may also be raised if rate of clearance from circulation is reduced as in impaired renal functions.

DIAGNOSTIC ENZYMOLOGY

In practice the most commonly used enzymes have a widespread distribution and they are not organ specific. Estimation of plasma enzyme activities is, therefore, of most value in confirming the diagnosis (e.g., myocardial infarction) or for monitoring the course of disease (e.g., viral hepatitis). All of the hundreds of different enzymes present in the human body are synthesised intracellularly. Certain enzymes are secreted, usually in an inactive form, and after activation, function within the extracellular fluids. Enzymes are classified in blood as:

1. **Plasma specific enzymes** such as proteases, procoagulants, thrombin, factor XII, factor X, and others
2. **Secreted enzymes** such as lipase, α-amylase, trypsinogen, cholinesterase, prostatic acid phosphatase
3. **Cellular enzyme** like lactate dehydrogenase (LD), alanine aminotransaminase (ALT), aspartate aminotransaminase (AST) and alkaline phosphatases (ALP) etc.

Enzyme estimations may be of value in the diagnosis and monitoring of:

1. Myocardial infarction (CK, LD and its isoenzymes, HBD and sometimes AST);
2. Liver diseases (transaminases, ALP, and sometimes γGT);
3. Bone diseases (ALP);
4. Prostatic carcinoma (tartrate-labile ACP);
5. Acute pancreatitis (α-amylase);
6. Muscle disorders (CK).

Distribution of diagnostically important enzymes is detailed in Table 50.1.

Table 50.1: Distribution of Diagnostically Important Enzymes

ENZYMES	PRINCIPAL SOURCES	CLINICAL APPLICATIONS
Acid phosphatase	Prostate, RBCs,	Carcinoma of prostate
Alanine amino-transferase	Liver, skeletal muscle, heart	Hepatic parenchymal disease
Aldolase	Skeletal muscle, heart,	Muscle diseases
Alkaline phosphatase	Liver, bone, intestinal mucosa, placenta, kidney	Bone diseases, hepatobiliary diseases
a-Amylase	Salivary gland, pancreas, ovaries,	Pancreatic diseases
Aspartate aminotransferase	Liver, skeletal muscle, heart, kidney, RBCs,	Myocardial infarction, hepatic parenchymal disease, muscle disease.
Cholinesterase	Liver,	Organophosphorus poisoning, suxamethonium sensitivity, hepatic parenchymal diseases,
Creatine kinase	Skeletal muscle, brain, heart, smooth muscle	Myocardial infarction, muscle diseases
Glutamate dehydrogenase	Liver,	Hepatic parenchymal diseases
γ glutamyl transferase	Liver, kidney,	Hepatobiliary disease, alcoholism
Lactate dehydrogenase	Heart, liver, skeletal muscle, RBCs, platelets, lymph nodes	Myocardial infarction, haemolysis, hepatic parenchymal diseases
5'-neucleotidase	Hepatobiliary tract	Hepatobiliary disease
Trypsin (ogen)	Pancreas	Pancreatic diseases

METHODS OF ESTIMATION

Enzymes are quantitated in terms of international units. **International unit** is defined as the quantity of enzyme that will catalyse the reaction of one micromole of substrate per min (see also UNITS IN CLINICAL ENZYMOLOGY on page 11). **Molar absorptivity constant** is the absorptive constant of an analyte at a given wave length under standard conditions of solvent, temperature, pH, path length and so forth. It is used for identification, quantitation and purity check of an analyte. Kinetic measurements are those where the enzyme activity is quantitated by measuring the amount of change of absorbance in a fixed time interval (ΔA). By these methods the amount of enzyme reagent required for each analysis can be reduced and the time is shortened. Coupled reactions are used to construct an enzyme analytical system for determining a particular compound and the specificity of coupled reaction modify the specificity of overall reaction. For example, in determination of glucose by hexokinase reaction, hexokinase will convert sugar other than glucose to their 6-phosphate esters. However, the indicator reaction used to monitor this change is catalysed by glucose-6-phosphate dehydrogenase, an enzyme that is highly specific for glucose only.

ENZYMES AS ANALYTICAL REAGENTS

The use of enzymes as analytical reagents offers the advantage of great specificity for the substance being determined. Enzymes with absolute specificity for the substance being estimated are clearly preferable for analytical use. Uricase, urease, and glucose oxidase are examples of highly specific enzymes used in clinically important assays.

ENZYMES OF CLINICAL IMPORTANCE

CREATINE KINASE (CK)

Principle: Creatine Kinase (CK) catalyses the reversible transfer of one phosphate to ADP thus forming ATP. The ATP reacts with glucose in the presence of hexokinase to form ADP and glucose-6-phosphate. Glucose-6-phosphate in turn reacts with NADP in the presence of glucose-6-phosphate dehydrogenase (G-6-PD) to form 6-phosphogluconate and NADPH. The rate of NADPH formation is proportional to the amount of CK and is measured photometrically at 340 nm. CK is most abundant in cells of cardiac and skeletal muscles. It also occurs in other tissues such as smooth muscle and brain.

Causes of raised plasma CK

1. **Artefactual** due to *in vitro* haemolysis.
2. **Physiological** during neonatal period.
3. **Marked** due to shock, circulatory failure, myocardial infarction, muscle dystrophies and rhabdomyolysis.
4. **Moderate** due to muscle injury, surgery, physical exertion, intramuscular injection, hypothyroidism, alcoholism, cerebrovascular accidents and head injury.

Reference range:
Men 38-195 U/L
Women 26-170U/L

Isoenzymes: CK molecule consists of M and B in various combinations forming three isoenzymes; BB (CK-1), MB (CK-2) and MM (CK-3). Most of the plasma enzyme activity is due to CK-MM. In myocardial infarction the activity of CK-MB rises to >5% of total CK activity.

LACTATE DEHYDROGENASE (LD)

Principle: Lactate dehydrogenase catalyses the reduction of pyruvate by NADH. The rate of decrease in concentration of NADH is proportional to the concentration of LD present in the sample. LD catalyses the reversible interconversion of lactate and pyruvate, It is

widely distributed in the body, with high concentrations in cells of cardiac and skeletal muscle, liver, kidney, brain, and erythrocytes. Measurement of total plasma LD activity is a non-specific marker of cell damage. Causes of raised plasma LD activity are:

1. **Artefactua**l due to *in vitro* haemolysis or delayed separation of plasma.
2. **Marked** increase due to circulatory failure, shock and hypoxia, myocardial infarction, megaloblastic anaemia, acute leukaemia, lymphoma, thalassaemia, myelofibrosis, haemolytic anaemia, renal infarction, or occasionally during rejection of renal transplant.
3. **Moderate** increase is due to viral hepatitis, malignancy of skeletal tissue, skeletal muscle disease, pulmonary embolism, and infectious mononucleosis.

Reference range: Serum: 225-450 U/L

Isoenzymes: Five isoenzymes can be separated by electrophoresis and referred to as LD_1 to LD_5. Certain patterns are of diagnostic importance:

1. Elevation of LD_1 and LD_2 (LD_1>LD_2, flipped ration) occurs in MI, megaloblastic anaemia and renal failure. LD_1 and LD_2 can use β-hydroxybutyrate, thus forms the basis of hydroxybutyrate (HBD) assays, which is an indication of LD_1.
2. LD_2 and LD_3 are raised in acute leukaemia; LD_3 main isoenzyme of malignancy.
3. Raised LD_5 occurs after damage to liver or skeletal muscle.

ASPARTATE TRANSAMINASE (AST)

Principle: AST catalyses the transfer of an amino group from aspartate to 2-oxoglutarate forming glutamate and oxaloacetate. The rate of decrease in concentration of NADH is proportional to the concentration of AST in the sample. AST is present in cells of cardiac and skeletal muscle, liver, kidney and erythrocytes. Causes of raised plasma AST activity are:

1. **Artefactual** due to *in vitro* haemolysis or delayed separation of plasma.
2. **Physiological** during neonatal period.
3. **Marked** increase is due to circulatory failure with shock and hypoxia, myocardial infarction, acute viral and toxic hepatitis.
4. **Moderate** increase occurs in cirrhosis, infectious mononucleosis, cholestatic jaundice, liver malignancy, skeletal muscle disease, post-trauma or post-surgery, and in severe haemolytic episode.

Reference range: Serum; 3-37 U/L

ALANINE TRANSAMINASE (ALT)

See in section on LIVER FUNCTION TESTS on page 328.

ALKALINE PHOSPHATASE (ALP)

See in section on LIVER FUNCTION TESTS on page 328.

Isoenzymes: Isoenzymes arising form cells of bone, liver, intestine and placenta can be separated by differences in their physical properties such as heat inactivity and mobility on electrophoresis, but is rarely required.

γ-GLUTAMYL TRANSFERASE (γGT)

See in section on LIVER FUNCTION TESTS on page 328.

α-**AMYLASE**

Principle: α-amylase catalyses the hydrolysis of blocked *p*-nitrophenylmaltoheptoside liberating oligomaltosides. The enzyme amyloglucosidase and α-glucosidase hydrolyse completely the oligomaltosides liberating *p*-nitrophenol. The rate of *p*-nitrophenol formation is proportional to the concentration of α-amylase in the sample. It is present at a higher concentration in pancreatic juice and in saliva and may be extracted from such other tissues as the gonads, fallopian tube, skeletal muscle, and adipose tissue. Causes of increase are:

1. **Marked** increase occurs in acute pancreatitis, severe glomerular impairment, severe diabetic ketoacidosis and perforated peptic ulcer.
2. **Moderate** increase is seen in acute abdominal disorders other than acute pancreatitis, perforated peptic ulcer, acute cholecystitis, intestinal obstruction, abdominal trauma, and ruptured ectopic pregnancy. Salivary gland disorders, mumps, salivary calculi, Sjögren's syndrome, morphine administration, severe glomerular dysfunction, myocardial infarction, acute alcoholic intoxication, and diabetic ketoacidosis.

Reference range: Serum; 25-109 U/L.

Macroamylasaemia: It is a benign condition in which high α-amylase level persists due to decreased renal clearance, despite normal renal functions. This is due either to the binding of α-amylase to high molecular weight proteins or formation of large α-amylase polymer molecules, which cannot pass through the glomerular membrane. This may cause confusion with the conditions having raised α-amylase activity.

LIPASE

Principle: Lipase catalyses hydrolysis of trioline to monoglyceride and oleic acid. The decrease in absorbance (turbidity) is measured at 340 nm and is proportional to the activity of enzyme in test sample.
Reference range: Up to 190 U/L
Interpretation: Following an attack of acute pancreatitis the activity of serum lipase rises to 2.0-10 times of normal within 2-12 hours. The activity also increases in ascitic fluid.

ALDOLASE

Principle: Aldolase catalyses the splitting of D-fructose-1,6-diphosphate to D-glyceraldehyde-3 phosphate and dihydroacetone phosphate, an important reaction in the glycolysis.
Reference range: Serum; 1.0-7.5 U/L
Interpretation:

1. Diagnosis of Duchenne muscular dystrophy (10-50 times elevation, carriers show slight to moderate increase)
2. Increased in dermatomyositis, polymyositis and limb-girdle dystrophy, Myocardial infarction (5-8 times), Viral hepatitis (7-20 times), Chronic granulocytic leukaemia, Megaloblastic anaemia

ACID PHOSPHATASE (ACP)

Total and Tartrate-labile ACP is used for the diagnosis and monitoring the treatment of prostatic carcinoma. It is being replaced by **prostate specific antigen** (PSA), a protein derived from prostate. This is more specific and sensitive for diagnosis and monitoring, however, it is also raised in similar circumstances to those affecting prostatic ACP and is more expensive.
Sampling: The value can rise two or three time the upper reference range by rectal examination. Marked rise occurs after prostatectomy. Therefore, sample should be taken at least three days after a rectal examination and after seven days of prostatectomy, to let the levels come back to baseline. Heparin inhibits the ACP activity.

PLASMA ENZYME PATTERNS IN DISEASES

Myocardial Infarction: The diagnosis is made by clinical presentation, electrocardiography and confirmed by characteristic changes in plasma enzymes activities. Plasma enzyme activities are raised in about 95% of cases. Degree of rise is a rough guide to the size of infarct. Plasma enzymes are normal until at least four hours after the onset of chest pain. The sample should not be taken unless this time has elapsed (Table 50.2 and Figure 50.1).

Table 50.2: Time sequence of changes in plasma enzymes after myocardial infarction.

Enzyme	Start to rise (hours)	Peaks at (hours)	Duration of rise (days)
CK (total)	4-6	24-48	3-5
AST	6-8	24-48	4-6
LD (HBD)	12-24	48-72	7-12

Other Cardiac Markers: Because of nonspecific nature of cardiac enzymes, and because of a time lag for their significant rise, newer cardiac markers are now being used increasingly. These include troponin T & I and myoglobin.

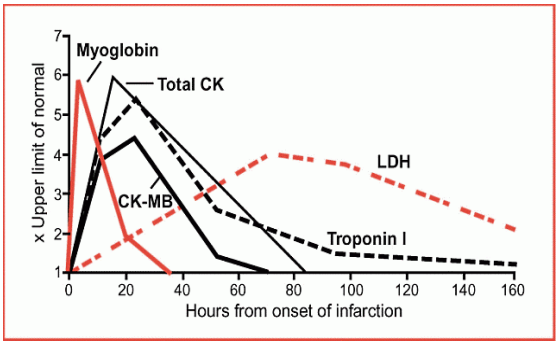


Figure 50.1: Time course of common cardiac markers

Liver Diseases: Plasma transaminases activity rises in hepatitis. In cholestasis there is predominant rise in ALP activity. Disproportional high γGT activity may suggest alcoholic liver disease. ALT activity more than AST suggests reversible alcoholic hepatitis, chronic persistent hepatitis, or early chronic active hepatitis. AST more than ALT may be due to cirrhosis or severe chronic active hepatitis. In hepatic invasion and infiltration AST is more sensitive and may be high despite a normal ALT activity. A mild fluctuating transaminase level will suggest chronic hepatitis due to HCV infection.

51. GASTRIC, PANCREATIC AND INTESTINAL FUNCTION TESTS

The gastrointestinal tract consists of oral cavity, oesophagus, stomach, small and large intestines. Important glands like pancreas, salivary glands, gall bladder, gastric and intestinal glands secrete enzymes and juices, which help in digestion of food. Main function of gastrointestinal tract is digestion, partial storage, absorption of ingested food and excretion of waste material.

GASTRIC FUNCTION

Stomach secretes **pepsin**, **hydrochloric acid** and **intrinsic factor**. Total volume of gastric secretions is 2800 ml. It digests proteins by converting large protein molecules into small polypeptides. Main disorder of gastric function is hypersecretion, which causes duodenal ulcer. Less common disorder is achlorhydria, in which gastric acid secretion is reduced.

GASTRIC FUNCTION TESTS

Tests of gastric function involving measurement of acid secretion have largely been replaced by endoscopic examination and biopsy. Stimulation of gastric secretion with measurement of gastric acidity is now done to test the completeness of section of vagus nerve. However following is a list of gastric function tests:

1. Measurement of acid:
   a. Basal secretion rate
   b. Peak and maximum acid output following pentagastrin stimulation
   c. Estimation of hydrochloric acid
   d. Estimation of total titrable acidity
2. Serum gastrin estimation
3. Endoscopy
4. Barium meal examination

Collection of Gastric Juice:

1. Patient should be having overnight fast.
2. A gastric tube should be passed in stomach.
3. Aspirate the gastric juice (resting juice).
4. Aspirate the stomach contents every 15 min for one-hour (basal secretion).
5. For stimulation test, give a stimulant e.g., pentagastrin 6 µg/kg body weight intramuscularly.
6. Aspirate stomach every 15 min for one hour (stimulated secretions).

ESTIMATION OF HYDROCHLORIC ACID IN GASTRIC JUICE

Principle: A known amount of gastric juice residue is titrated with 0.1 mol/L sodium hydroxide to a *p*H of 3.5 using a *p*H meter or Toepfer's reagent as indicator.

Reagents:

1. Sodium hydroxide 0.1 mol/L. Dissolve 4g sodium hydroxide and make up to 1 L with distilled water.
2. Toepfer's reagent: Dissolve 0.5 g diethylaminoazobenzene in 100 ml ethanol.

Procedure:

1. Pipette 5 ml gastric juice into a clean titration vessel. If it contains food particles or mucus, centrifuge the sample.
2. Check the *p*H of the sample with *p*H meter. If *p*H is above 3.5 then no free acid is present. Alternatively, add two drops of Toepfer's reagent. If yellow colour develops then *p*H is about 3.5 and no free acid is present. Such specimen need not be titrated. If *p*H of gastric juice is below 3 or a red colour develops after the addition of Toepfer's reagent then free acid is present and proceeds for titration.
3. Titrate the sample with NaOH (0.1 mol/L) to a *p*H of 3.5 using a *p*H meter or add two drops of Toepfer's reagent. Titrate to salmon colour.

Calculation:

$$\text{Free HCl (mmol/l)} = \frac{\text{ml NaOH} \times 0.1 \times 1000}{\text{ml gastric specimen tritrated}}$$

As 5 ml gastric specimen is titrated so free HCl (mmol/L)=ml of NaOH x 20

$$\text{Basal acid output (mmol/h)} = \frac{\text{mmol free HCl/l} \times \text{vol of specimen (ml)} \times 60}{1000 \times \text{collection period (min)}}$$

Maximum acid output/hour of four 15 min post stimulation specimen. Average acid output value and this gives maximum acid output/L (MAO/L)

Peak acid output: Calculate the acid output for six 15 min post stimulation specimens. Select the two specimens with the highest acid output. Take the mean of the two values and this gives peak acid output per hour (PAO/h).

ESTIMATION OF TOTAL TITRATABLE ACIDITY IN GASTRIC RESIDUE

Titrate 5 ml fasting gastric juice to a *p*H of 7.0 using a *p*H meter or phenol red indicator. Calculate total titrated acidity in the same way as for free HCl.

Reference ranges

1. Volume:	<50 ml
2. pH:	1.5 – 3.5
3. Basal acid out put (BAO):	0.5 mmol/L
4. Total titrated acidity	10-60 mmol/L
5. Peak acid out put (PAO):	5-20 mmol/L
6. BAO/PAO percentage:	<20%

Interpretation

	Duodenal ulcer	Zollinger-Ellison syndrome	Gastric ulcer
BAO	5-15 mmol/L	>20 mmol/L	N
PAO	20-58 mmol/L	>60 mmol/L	N
BAO/PAO	40-80%	>60%	N

ROLE OF GASTRIN IN CONTROL OF SECRETIONS

Gastrin is a peptide hormone consisting of 34 amino acids (G-34). It causes stimulation of gastric acid secretion, pepsin secretion, gastric motility and growth of gastric mucosa. Increased vagal discharge, gastric distension, and amino acids peptides in the stomach and calcium in blood stimulate its secretion. It is inhibited by gastric acidity and gastrointestinal hormones e.g., secretin.

Reference Range and Interpretation:

Normal	up to 100 ng/L
Duodenal ulcer	100-200 ng/L
Zollinger-Ellison Syndrome	>200 ng/L

PANCREATIC FUNCTIONS

The pancreas has both endocrine and exocrine functions. Endocrine functions include secretion of insulin, glucagon and pancreatic polypeptide. Exocrine secretion is alkaline and it includes secretions of α-amylase for carbohydrate digestion, trypsin, chymotrypsin and carboxypeptides for protein digestion and lipase for fat digestion. In addition, bile duct contains bile, which is secreted in small intestine through the pancreatic duct.

Control of Secretions

It is controlled by secretin a 27 amino acid polypeptide hormone secreted by small intestine in response to presence of acid in small intestine and stimulates alkaline secretion. Cholecystokinin (CCK) a 33 amino acid polypeptide hormone secreted by small intestine in response to presence of acid. It stimulates the secretion of exocrine pancreatic enzymes like trypsin, chymotrypsin, carboxypeptidase, lipase and α-amylase. Tests of pancreatic function are of two types:

1. **Direct Tests**: In direct tests oral intubation of the patient is required. These tests are unpleasant for the patient and the patient should be explained the procedure before performing the test. These include:
   a. Secretin-Cholecystokinin test
   b. Lundh test
2. **Indirect Tests**: These test are performed on serum or urine and include:
   a. Fluorescin Dilaurate test
   b. ^{14}C-PABA test (*p*-aminobenzoic acid labelled with radioactive carbon)
   c. Serum α-amylase
   d. Serum lipase

Serum α-amylase and lipase while having little value in malabsorption is useful as acute pancreatic function test. Serum α-amylase (see section on α-AMYLASE on page 350) and lipase levels (LIPASE on page 351) are increased in acute pancreatitis.

SECRETIN-CHOLECYSTOKININ TEST

Procedure: Patient should be in a fasting state. Nasogastric intubation with a double lumen should be done in such way that one orifice is in the stomach and other in the duodenum near the opening of pancreatic duct.

'0' min: Aspirate and discard resting duodenal juice. Then administer 1 CU/kg body weight secretin, prepared at concentration of 10 CU/ml in normal saline, slow intravenously. Collect the duodenal juice at 10 min intervals for 30 min.

30 min: Give 1 CU/kg body weight cholecystokinin (CCK) slow intravenously. Collect duodenal juice at 10 min intervals for another 30 min. Keep all samples at 2-8°C before analysis. Measure the volume of duodenal juice. Analyse the samples immediately for bicarbonate, α-amylase and tryptic activity.

Reference values

Peak bicarbonate concentrated:	90 mmol/L
Peak tryptic activity:	30 IU/ml
Peak α-amylase activity:	270 IU/ml

LUNDH TEST

Principle: In this test pancreatic secretion is stimulated physiologically by giving test meal containing corn oil, skimmed milk powder and dextrose.

Procedure: Pass a nasogastric tube. Give test meal containing 19 g corn oil, 15 g skimmed milk powder and 40 g dextrose through the gastric tube. Duodenal juice is aspirated for two hours and is analysed for tryptic activity.

Interpretation: α-Amylase and tryptic activities

are decreased in:
1. Surgical removal of pancreas
2. Pancreatic duct obstruction
3. Cystic fibrosis

FUNCTIONS OF SMALL INTESTINE

Small intestine performs the function of digestion and absorption of carbohydrates, proteins and fats. In addition it is responsible for absorption of fat and water-soluble vitamins, iron, calcium and magnesium etc. The tests of intestinal function include:

1. Tests of carbohydrate absorption
   a. Xylose absorption test
   b. Glucose tolerance test
   c. Lactose tolerance test
   d. Reducing substances and *p*H in stool
2. Tests of protein absorption
   Nitrogen in stool
3. Tests of fat absorption
   a. Faecal fat estimation
   b. Plasma triglyceride and cholesterol
   c. Vitamin A absorption
   d. ^{14}C-labelled triglyceride test

Among these tests only xylose absorption test, faecal fat estimation, cholesterol and triglyceride estimation are performed.

XYLOSE ABSORPTION TEST

Principle: Xylose, a pentose is not commonly present in blood, does not require digestion and is actively absorbed by small intestine. It is not metabolised by body and is excreted in urine. Xylose in diluted urine and protein free filtrate of blood is treated with *p*-bromoaniline in an acidic medium. When heated, xylose is dehydrated to furfural, which gives pink colour with *p*-bromoaniline. Thiourea reduces the formation of interfering coloured compounds.

Sample collection
1. Patient should be having an overnight fast.
2. Empty the bladder completely at the start of test.
3. Give 5 g D-xylose in 250 ml of water.
4. Collect 3 ml blood in EDTA-sodium fluoride tube 2 hours after giving xylose.
5. Collect all urine samples within 5 hours of giving xylose.

Reagents:
1. Zinc sulphate ($ZnSO_4$ 7H2O): 5% in distilled water.
2. Barium hydroxide $Ba(OH)_2$ $8H_2O$: Dissolve 23.7 g of barium hydroxide in water and dilute to 500 ml. Boil for a few min. Allow to cool and filter.
3. *p*-bromoaniline reagent 2%: Prepare a saturated solution of thiourea in 100 ml glacial acetic acid and dissolve 2 g *p*-bromoaniline.
4. Xylose stock standard (0.2g%). Dissolve 200 mg of xylose in 100 ml saturated benzoic acid solution.
5. Xylose working standard. Dilute the stock standard 10 & 20 fold with saturated benzoic acid solution. These contain 200 and 100 mg xylose/L respectively

Procedure (urine)
1. Dilute total urine to 1000 ml with distilled water.
2. Take 1 ml of this diluted urine and make total volume to 10 ml with distilled water. Proceed as follows:

	Test	Blank
Finally diluted urine	1 ml	1 ml
p-bromoaniline reagent	5 ml	5 ml

3. Place the test tubes in water bath at 70°C for 10 min, then cool and leave in the dark for 70 min. The blank should remain at room temperature and in light.
4. Put up the standard and standard blank in the same manner, using 1 ml of 200 mg/L xylose standard instead of urine.
5. Read the unknown test against its own blank at 530 nm and the standard against its own blank.

Calculation $\text{xylose excreted (g)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 2$

Interpretation: More than 1.2 g of xylose should be excreted in 5 hours in normal persons. Results lower than this indicates some degree of malabsorption, if the renal functions are normal.

Procedure (blood):
Prepare a Somogyi filtrate (PROTEIN FREE FILTRATES on page 50). Proceed exactly as for diluted urine, taking 1 ml of supernatant or filtrate. Use 1 ml 100 mg/L xylose standard.

Calculation: $\text{Blood Xylose (mg/L)} = \frac{\text{Abs of test}}{\text{Abs of std}} \times 1000$

Reference range: In normal subjects the blood xylose level should be above 200 mg/L.

Interpretation: Decrease absorption is seen in coeliac disease, Tropical sprue, Intestinal resection, acute diarrhoea, Blind loop syndrome and Massive bacterial overgrowth in small intestine. Increased absorption is seen in Gastrectomy. The test is invalid in presence of impaired renal functions and generalised oedema.

Sources of non-analytical errors:
1. Low renal threshold.
2. Wrong collection of urine specimen.
3. Improper time of blood sample collection

STEATORRHOEA

Steatorrhoea is passage of excessive fat in stools of more then 7 g/day. It is one of the earliest features of malabsorption. Major causes of steatorrhoea are:

1. Lipase deficiency
   a. Chronic pancreatitis
   b. Pancreatic resection
   c. Carcinoma of pancreas
   d. Cystic fibrosis
2. Bile salts deficiency
   a. Biliary obstruction
   b. Chronic liver disease
   c. Disease/resection of terminal ileum
   d. Blind loop syndrome
3. Defects in intestinal wall
   a. Coeliac disease
   b. Tropical spure
   c. Small bowel resection
   d. Regional ileitis
   e. Abetalipoproteinaemia
   f. Intestinal lymphoma
   g. Amyloid, scleroderma etc.
4. Miscellaneous
   a. Post gastrectomy
   b. Zollinger-Ellison syndrome
   c. Carcinoid syndrome
   d. Diabetes mellitus
   e. Parasitic infestation
   f. Whipples disease

FAECAL FAT ESTIMATION

This is the test for estimation of residual fat in the faeces. After oral intake and digestion, almost all fat is absorbed. Most of fat normally excreted in faeces is derived from enterocytes.

Sample collection: Patient should be on a diet containing 50-150g of fat for three days. Stool specimen should be collected for at least five days in pre-weighed tight lid containers. Carmine markers can be used on day 0 and day 5. Any obvious foreign matter should be removed from the specimen prior to analysis.

Principle: A pre-weighed emulsified stool specimen is acidified to decrease the ionisation of fatty acids. The lipids including less polar non-esterified fatty acids are extracted from stool specimen with organic solvents, the supernatant evaporated and the residue quantitated by gravimetry.

Reagents

1. Ethyl ether: protect from heat light and atmospheric air. It should be peroxide free.
2. Analytical grade *n*-heptane
3. Ethyl alcohol absolute and 95%
4. Solvent A: Mix the equal volume of *n*-heptane, ethyl ether and ethyl alcohol. Prepare fresh.
5. Solvent B: Mix equal volumes of *n*-heptane, ethyl ether, water and 95% ethanol. Use upper phase for extraction. Prepare fresh.

Procedure

1. Weigh the specimen with the container. Subtract the container weight from total weight to get net weight (W).
2. In a well-ventilated safety cabinet open the container and if faeces are firm, dilute with water twice the weight of faeces.
3. Tightly close the lid of container and shake vigorously for 10 min.
4. Open the container in a fume cupboard immediately. Transfer 3 g of faecal emulsion into a 50-ml screw capped centrifuge tube.

EXTRACTION

1. Add four drops of concentrated HCl to the specimen and mix it.
2. Pipette 20 ml of solvent A to the acidified faecal homogenate. Tightly cap it and mix it for 5 min.
3. Centrifuge for 10 min at 3000 revolutions per min. Aspirate the supernatant into a preweighed 100-ml beaker.
4. Add 20 ml of solvent B to supernatant and re-extract the fats. Repeat this procedure once again. Add each extract to beaker in step 3.
5. Evaporate the combined extracts to dryness.
6. Weigh the beaker and subtract the weight of empty beaker to obtain the weight of lipid residue.

Calculation

Faecal lipid (g)=Wt of lipid residue X dilution X wt of faeces

Reference range: Normal 24 hours fat excretion is up to 5 g. (Fat excretion of more then 7 g/day indicates steatorrhoea.

OTHER TESTS FOR FAT ESTIMATION

1. Microscopic examination of stool for fat globules.
2. ^{14}C triolin breath test

INVESTIGATION OF SUSPECTED MALABSORPTION SYNDROME

Malabsorption is usually suspected if patient gives the history of prolonged diarrhoea of unknown cause and has features of malnutrition. Patient may have the history of long term antibiotic intake, intestinal surgery or travels to tropics. The clinical laboratory has a limited role in investigation of malabsorption because of availability of other investigations.

52. INBORN ERRORS OF METABOLISM

Many inherited diseases are due to the genetically determined absence or modification of specific proteins. The clinical features of inherited metabolic diseases stem directly from the metabolic abnormalities. Although individually these conditions are rare, they are of considerable significance due to their potentially disastrous consequences. Many of them may in some cases be ameliorated if an early diagnosis is made and the appropriate treatment is instituted.

CLASSIFICATION

For practical purposes, the metabolic disorders may be divided into 9 groups.

1. Disorders of amino acid metabolism associated with neurological symptoms.
2. Disorders in amino acid transport
3. Disorders of carbohydrate metabolism
4. Lysosomal enzyme disorders
5. The mucolipidoses and disorders in glycoprotein metabolism.
6. Disorders manifested by intermittent metabolic acidosis (organic acidurias)
7. Disorders of lipid metabolism
8. Disorders of metal metabolism
9. Disorders of purine metabolism

AMINO ACIDURIAS

The renal threshold for plasma amino acids is high, so that only small amounts of amino acids are normally found in urine. The disorders characterised by the presence of increased amounts of amino acids in the blood and urine may be due to an inborn error of metabolism, severe liver diseases or the result of a disorder of tubular transport mechanism.

CARBOHYDRATE DISORDERS

Some of the common carbohydrate disorders are described briefly:

Intestinal lactase deficiency is a common problem leading to cramping abdominal pain and osmotic diarrhoea after ingestion of lactose containing food. The uncommon lactose, fructose and sucrose imbalance causes severe illness with vomiting in young infants. Lactose and hereditary fructose intolerance may cause liver dysfunction and renal tubular damage. Removal of the sugar from the diet will alleviate the difficulty. **Pentosuria** is benign. **Glycosuria** and **hyperglycaemia** may be seen with central nervous system disorders; brain tumours or haemorrhage, hypothalamic disease, asphyxia and disturbance of metabolism. Glycosuria without hyperglycaemia is usually associated with renal tubular dysfunction. True inherited renal glycosuria is uncommon, it is associated with reduced glucose reabsorption. Galactose is found in the urine in genetic disorders of galactose metabolism associated with a deficiency of either galactokinase or in the classic disease, galactose-1-uridyl transferase. The diseases are transmitted as autosomal recessive.

LYSOSOMAL DISORDERS

Lysosomes are cytoplasmic organelles, which enclose an acidic environment containing numerous enzymes capable of hydrolysing most biological macromolecules. A major function of the lysosome is degradation of used macromolecule related to normal turnover and tissue remodelling The lysosomal diseases emphasize the physiological significance of this disposal role and include most of the lipid storage disorders, the mucopolysaccharidoses, the mucolipidoses, glycogen storage disease and many others. A lysosomal storage disease is usually suspected on the basis of progressive neurological dysfunction, visceromegaly, and skeletal dystosis. Progressive or degenerative disease is the hallmark of these disorders.

MUCOPOLYSACCHARIDOSES (MPS)

Mucopolysaccharidoses (MPS) are group of diseases characterised by excessive amount of mucopolysaccharide storage in organs. It is a syndrome of mental and physical retardation, multiple skeletal deformities, hepatomegaly, and clouding of the cornea. Seven groups and some subdivision have been described according to the clinical features and specific enzyme deficiencies. The mucopolysaccharides and their partially degraded forms excreted in large amounts in urine are dermatan heparin and keratan sulphates and in type VII, chondrotin sulphate.

LEUKODYSTROPHIES

These are number of rare brain diseases, occurring mainly during childhood. There is

diffuse demyelination of the white matter of the cerebral hemispheres forming cerebroside sulphuric acid esters (sulphatides). These esters may be seen as granules in urine, which stain brown with orthotoluidine blue. The stain test is only a screening procedure but when properly interpreted and positive, is suggestive of but not diagnostic of hereditary metachromatic leukodystrophies.

PURINE /PYRIMIDINE DISORDERS

Gout is a term representing a familial heterogeneous group of diseases found exclusively in humans (see also PURINE AND URATE METABOLISM on page 341). It is manifested by:

1. An increase in the serum urate concentration
2. Recurrent attacks of characteristic type of acute arthritis
3. Tumour-like tophi in and around points of extremities
4. Renal disease/urate nephrolithiasis.

Abnormal purine metabolism in Lesch-Nyhan disease is due to deficiency of hypoxanthine-guanine-phosphoribosyl-transferase (HGPRT) and affects male children. It affects the central nervous system and causes hyperuricaemia, gout; stones and urate neuropathy.

SCREENING FOR INBORN ERRORS OF METABOLISM

Urine has been used for many years to screen for metabolic diseases. These include use of routine urine analysis and simple screening tests. Urine must be processed for metabolic diseases as soon as received in the laboratory. For example, phenylketonuria (PKU) is tested for phenylpyruvic acid, which is unstable at room temperature. In case testing is not possible immediately, urine must be refrigerated soon after voiding. The following step-by-step approach is suggested:

1. Routine urine analysis
2. Ferric chloride test
3. Benedict's test
4. MPS test
5. DNPH test
6. Nitroprusside cyanide test
7. Metachromatic staining
8. Amino acid test

URINE ANALYSIS

Routine urine examination is performed (for details see URINE EXAMINATION on page 77). It is very important in deciding the subsequent approach. In microscopy, crystals need special attention: urate stone formation, Lesch-Nyhan syndrome, gout and urate nephropathies; cystine in cystinuria and other tubular diseases, tyrosine in tyrosinosis.

FERRIC CHLORIDE TEST

Ferric chloride test is nonspecific. It will give colour reactions in several amino acid disorders, with other metabolites and drugs. The ferric iron chalets with the enol group and will produce colour with ketoacids from corresponding amino acids (Table 52.1), PKU, alkaptonuria, histidinaemia, tyrosinosis, and, maple syrup urine disease may cause colour reaction in urine. Urine must be fresh.

Table 52.1: Ferric chloride test in urine

Substance or disease	Colour
Acetoacetic acid	Red or red brown
Bilirubin	Blue green
Homogentisic acid	Blue or green; fades quickly
α-hydroxyphenylacetic acid	Mauve
o-hydroxyphenyl pyruvic acid	Red browns turned to green or blue then fades to mauve
p-hydroxyphenyl pyruvic acid	Green or blue green
Imidazole pyruvic acid	Green or blue green
α-ketobutyric acid	Purple, fades to red brown
Maple syrup urine disease	Blue
Melanin	Grey precipitate; turns black
Phenylpyruvic acid	Green or blue green; fades to yellow
Pyruvic acid	Deep gold yellow or green
Xanthurenic acid	Deep green; later brown
Drugs	
Aminosalicylic acid	Red-brown
Antipyrines and acetophene-tidines	Red
Cyanates	Red
Phenol derivatives	Violet
Phenothiazine derivatives	Purple pink
Salicylates	Stable purple

Reagents: Ferric chloride 10% (10g/100 ml in distilled water) store in refrigerator.

Procedure: Add 2-4 drops of reagent to 1 ml urine. PKU is indicated by green or blue green colour appearing in 90 sec and fading again in the same period of time. Other substances will give colours according to Table 52.2.

BENEDICT'S TEST

For principle, reagent and procedure see section on Benedict's test: in URINE EXAMINATION on page 80. Positive reaction is usually obtained in patients with galactosaemia, fructose intolerance and in some children excreting large amounts of tyrosine and its metabolites. An enzymatic reagent strip such as Clinistix is used to identify glucose. Urine thin layer chromatography (see also THIN LAYER CHROMATOGRAPHY on page 40) identifies other reducing sugars such as lactose, fructose, galactose and pentose. If

this is negative, the reducing substance is not sugar but is most likely a drug or drug metabolite.

MUCOPOLYSACCHARIDE (MPS) TEST

A simple screening test involves 'Eye balling' of the metachromasia produced, when urine is dried on filter paper impregnated with azure A dye treated with wash solution.

Reagents:

1. Test paper (Whatman #1) impregnated with 0.5% azure A dye made in distilled water.
2. Wash solution: Mix 29 ml methanol, 0.1 ml glacial acetic acid to water to make 200 ml.

Procedure: Use fresh, random or 24 hour refrigerated (without preservative) urine. Cloudy urine must be filtered first or centrifuged. Place one drop of urine in the middle of test paper. After 3 min, transfer to petri dish with wash solution. Rinse for 20 min, remove and blot dry.

Results: Positive reaction is indicated by a distinct purple colour where urine has been applied. Negative reaction gives only pale-blue background.

DINITROPHENYLHYDRAZINE (DNPH) TEST

This test indicates the presence of α-keto amino acid in the urine. Insoluble hydrazones form from the reaction of carboxyl groups with dinitrophenyl hydrazine. A positive result is seen with maple syrup urine disease and possibly in phenylketonuria (phenylpyruvic acid), histidinaemia (imidazole pyruvic acid) and methionine malabsorption (Oasthouse syndrome). The test is positive with ketonuria due to inherited diseases. A preliminary screening test for ketones should be done.

Reagents: 2,4 Dinitrophenyl hydrazine 0.5% in 2N HCl made by diluting 168 ml concentrated HCl to 1L.

Procedure: To 1 ml centrifuged fresh urine add 0.2 ml reagent drop by drop. A definite yellowish white precipitate forming within one min represents a positive reaction.

NITROPRUSSIDE-CYANIDE TEST

The diagnosis of homocystinuria suggested by the appearance of the patient may be confirmed by a positive urinary nitroprusside-cyanide test, an increased urinary excretion of homocystine and by an elevated plasma methionine.

Procedure: To 5 ml of urine add several drops of concentrated ammonium hydroxide and 2 ml 5% solution of sodium cyanide. After 5-10 min, a few drops of a 5% solution of sodium nitroprusside are added. A deep purple colour indicates presence of large amounts of cystine and acetone and glutathione.

Table 52.2: Screening for inborn errors of metabolism

Disease	Ferric chloride test	Benedict's test	MPS test	DNPH test	Amino acid test	Nitroprusside test	Meta chromatic granules stain
Phenylketonuria	+	+	-	+	+	-	-
Tyrosinuria	+	+	-	+	+	-	-
Tyrosinosis	+	+	-	+	+	-	-
Histidinaemia	+	-	-	+	+	-	-
Maple syrup urine disease	+	-	-	+	+	-	-
Lowes syndrome	-	-	-	+	+	-	-
Hartnup disease	-	-	-	-	+	-	-
Wilson's disease	-	-	-	-	+	-	-
Arginosuccinic aciduria	-	-	-	-	+	-	-
Hyperglycaemia	-	-	-	+	+	-	-
Propionic acidaemia	+	-	-	+	+	-	-
Methylmalonic aciduria	+	-	-	+	+	-	-
Homocystinuria	-	-	-	-	+	+	-
Cystathioninuria	-	-	-	-	+	+	-
Cystinuria	-	-	-	-	+	+	-
Glutathioninuria	-	-	-	-	+	+	-
Lead poisoning	-	+	-	-	+	-	-
Galactosaemia	-	+	-	-	+	-	-
Fructosuria	-	+	-	+	+	-	-
Metachromatic leukodystrophy	-	-	-	-	-	-	+
Mucopolysaccharidoses	-	-	+	-	-	-	-

METACHROMATIC STAINING

There are a number of rare cases of childhood brain diseases due to diffuse demyelination of cerebral hemispheres. One subgroup is called metachromatic leukodystrophy. It also shows renal involvement and metachromatic granules in the urinary sediment. These can be stained with toluidine blue.

Reagent: Toluidine blue 2% in distilled water.

Procedure: Mix 2 drops of toluidine blue to the sediment of fresh urine. Transfer a small quantity to a microscope slide and examine for brownish granules, 3-5 μm in diameter. The golden brown granules are found free, in casts, within cells or in clusters in sediments in patients of metachromatic form of diffuse cerebral sclerosis. Urine of these patients is deficient in arylsulfatase activity.

AMINO ACIDS TEST

In many metabolic disturbances it is not the total concentration of amino acids that is of clinical importance, but rather the altered concentration of one amino acid or a group of related amino acids. In many such instances, the abnormalities can be readily detected by simple screening tests of urine using chromatography (paper or thin layer) or high voltage electrophoresis.

SUMMARY

Table 52.2 summarises some of the inborn errors of metabolism that can be detected by simple screening tests.

53. HORMONE SYSTEMS OF THE BODY

Hormone is a Greek word meaning to excite, to set emotion, to arouse. A **hormone** has been traditionally defined as a chemical substance that is produced by a specialised ductless gland, secreted directly into the blood stream and carried to a distant target organ where it elicits regulatory response. However, now it is known that glandular tissues can also secrete a hormone and mediums other than the blood circulation can transport it. Moreover, it can act in close proximity to neighbouring cells (**paracrine** action) and on the cell in which it is produced (**autocrine** action). A hormone may be a protein, polypeptide or steroid derived from amino acids (tyrosine) and fatty acids (prostaglandin). They possess a high degree of structural specificity. A slight alteration in the molecular structure may bring significant changes in its physiological activity. Some of the hormones have negative feed back control i.e., the rise in concentration of certain hormone in the blood inhibits the secretion of that hormone which causes its secretion. They perform important functions like growth, body metabolism, electrolyte homeostasis, sexual functions, and regulation of carbohydrate, fat and protein metabolism etc. Their deficiency or excess results in a variety of disorders. Important hormonal disorders are listed in Table 53.1. The production of abnormal hormones, resistance of target tissue to hormone action and abnormalities of hormone action itself can also cause few disorders.

A few endocrine glands and the important hormones secreted by them are listed below:

Hypothalamus
Thyrotropin-releasing hormone (TRH)
Gonadotropin releasing hormone (GnRH)
Corticotropin-releasing hormone (CRH)
Growth hormone releasing hormone (GHRH)
Somatostatin (SS)
Prolactin releasing factors
Prolactin inhibiting factors

Anterior pituitary
Thyroid stimulating hormone (TSH)
Adrenocorticotrophic hormone (ACTH)
Follicle stimulating hormone (FSH)
Leutinizing hormone (LH)
Growth hormone (GH)
Prolactin (PRL)

Table 53.1: Common clinical features associated with hormone deficiency and excess

Hormone	Clinical features
FSH/LH deficiency	Amenorrhoea, and infertility in women, impotence in men and delayed puberty in children
FSH/LH excess	True precocious puberty
GH deficiency	Dwarfism in children.
GH excess	Acromegaly in adults, gigantism in children
TSH deficiency	Secondary hypothyroidism
TSH excess	Secondary hyperthyroidism
Prolatin deficiency	Suppressed lactation and breast atrophy in women
Prolactin excess	Galactorrhoea, amenorrhoea and infertility in women. Gynaecomastia and impotence in men
ACTH deficiency	Secondary hypocortisolism
ACTH excess	Cushing's disease
T3, T4 deficiency	Hypothyroidism (myxoedema)
T3, T4 excess	Thyrotoxicosis (Grave's disease)
PTH deficiency	Hypoparathyroidism
PTH excess	Hyperparathyroidism
Insulin deficiency	Diabetes mellitus
Insulin excess	Insulinoma
Cortisol deficiency	Addison's disease
Cortisol excess	Cushing's syndrome
Aldosterone deficiency	Hypoaldosteronism
Aldosterone excess	Hyperaldosteronism
Catecholamine excess	Pheochromocytoma
Testosterone deficiency	Male hypogonadism (and infertility)
Oestrogen/ progesterone deficiency	Female infertility

Posterior pituitary
Antidiuretic hormone (ADH)
Oxytocin

Thyroid gland
Thyroxine (T4)
Tri-iodothyronine (T3)

Adrenal cortex
Cortisol
Aldosterone
Androgens

Adrenal medulla
Adrenaline
Noradrenaline

Pancreas
Insulin
Glucagon

Parathyroid gland
Parathormone (PTH)
Calcitonin

Ovary
Oestrogen
Progesterone

Testis
Testosterone
Dihydrotestosterone (DHT)

Almost all of these hormones can be assayed in the blood or some of their metabolites in urine. However, their assayed values may not be diagnostic of an endocrine disorder. Because the levels of hormones in blood are subject to gross variation depending upon the physiological state of the body at the time of sampling. Best examples are diurnal variations in serum cortisol level and variations in female sex hormones in relation to stage of menstrual cycle. For accurate diagnosis, it is important to induce or suppress secretion from the endocrine gland by an appropriate physiological or pharmacological stimulus. Therefore, one or more of the following methods may test hormone system of the body:

- Assay of hormones in blood
- Assay of hormone metabolites in urine
- Dynamic function tests

ESTIMATION OF HORMONES IN BLOOD

A variety of analytical methods are available for estimation of hormones in blood. Specific equipment and reagent kits are commercially available for each of these. While using a method, the instructions of the manufacturer of analytical system should be strictly followed. Most common of these are:

Radioimmunoassay: It is a competitive binding assay in which hormone in the sample competes with the same hormone labelled with radioactive isotope. This method has good sensitivity and specificity and is relatively cheap. For details see section on RADIOIMMUNOASSAY on page 412.

Immunoluminometric assays: This is a two-site solid phase immunoluminometric assay (sandwich principle). For details see section on LIA-MAT-300 in AUTOMATION IN CHEMICAL PATHOLOGY on page 62.

Chemiluminescence Immunoassay: This is a competitive immunoassay. The principle of the procedure is that it utilises specific antibody coated polystyrene beads as the solid phase for incubation, wash and signal development processes. After the sample is incubated with alkaline phosphatase-labelled reagent, the reaction mixture is separated from the bead by centrifugation. The bound label is quantified by the chemiluminescent substrate reacting with the alkaline phosphatase label bound to the bead. Chemiluminescence immunoassay is more sensitive than the above techniques and has increased detection limits of hormones. It is however more expensive.

ESTIMATION OF HORMONE METABOLITES IN URINE

Some of the hormones are difficult to measure in the plasma because of their circadian rhythm. The metabolites of these hormones are concentrated and excreted in the urine. The urinary estimation of these metabolites, therefore, may be more useful in determining hypo or hyperfunction of the endocrine gland. The commonly performed urinary metabolites include the following:

URINARY 17-KETOSTEROIDS ESTIMATION

17 ketosteroids are the metabolic end products of adrenal androgens and constitute about 75% of total output of androgens by the adrenal cortex. Their estimation is used to investigate the cases presenting with hirsutism and virilisation.

Principle: Conjugated 17-ketosteroids are hydrolysed by sulphuric acid in the presence of formaldehyde and extracted with ethylacetate. The extract is washed with alkali to remove phenolic steroids and then with a salt solution that removes traces of alkali. The purified extract is evaporated to dryness in water bath. Colour is developed in aqueous medium by modified Zimmermann reaction using a quaternary ammonium salt. Photometric readings are made at 530 nm.

URINARY 17-HYDROXYSTEROID ESTIMATION

17-hydroxysteroids are the metabolites of the adrenal corticosteroids. Although plasma and urinary cortisol are more specific but in most cases disturbances in corticosteroid hormones are reflected in the urinary excretion of 17-hydroxysteroids.

Principle: This test is based on the " Porter and Silver colour reaction". Corticosteroids react with phenyl hydrazine in the presence of alcohol and sulphuric acid to form a yellow coloured pigment proportional to the amount of 17-hydroxysteroids in the urine.

Reference range

Children up to one year:	0.5-1.0 mg/day
Adult male:	3-10 mg/day
Adult female:	2-8 mg/day

URINARY VMA ESTIMATION

Vanillylmandelic acid (VMA) is one of the metabolites of catecholamines, mainly produced in the brain, adrenal medulla and the sympathetic neurons. Measurement of VMA is primarily used for the diagnosis of catecholamines secreting neurochromaffin tumour such as phaeochromocytomas, paragangliomas and neuroblastomas. These tumours may produce excessive amounts of catecholamines or catecholamine metabolites. Thus the urinary 24h excretion of VMA is markedly increased. The *p*H of the urine is kept low during collection by placing 10 ml of concentrated HCl into a suitable container (Dark brown bottle).

Principle: VMA is retained by anionic exchange resin, being eluted thereafter once the interfering substances are washed away. The VMA is quantified photometrically at 340 nm as vanillin after peroxidase oxidation under alkaline conditions. Other method of VMA estimation includes paper chromatography, thin layer chromatography HPLC, and High voltage electrophoresis.

Reference Range:

Children: 5-16 µmol/day
Adults: 7-33 µmol/day

THE PITUITARY GLAND

The pituitary gland is sometimes called the **master gland** of the endocrine system, because it controls the functions of the other endocrine glands. The pituitary gland is of the size of a pea, located at the base of the brain. The gland is attached to the hypothalamus (a part of the brain that affects the pituitary gland) by nerve fibres. The pituitary gland itself consists of the anterior lobe, the intermediate lobe and the posterior lobe. Each lobe of the pituitary gland produces certain hormones.

ANTERIOR PITUITARY

The measurement of the basal (resting) level of individual hormones often gives equivocal results as the pituitary has a large functional reserve. If a hormone deficiency is suspected the pituitary gland is stimulated to produce excessive hormone secretion, investigating the pituitary reserve (**Stimulation tests**). If there is excessive production of a hormone, it is inhibited by **suppression test**. It should be remembered that excessive secretion by tumour tissues is autonomous.

GROWTH HORMONE SUPPRESSION TEST

The test is of value in confirming the presence of active acromegaly or gigantism, particularly in the early stages.

Principle: In the presence of either active acromegaly or gigantism, the normal suppression of growth hormone (GH) by food or glucose does not occur.

Preparation: This is as for the oral glucose tolerance test (OGTT). The patient should not be receiving GH-stimulating drugs.

Procedure: This is as for the OGTT.

Normal response: The normal response is for serum GH to be suppressed to <3 mIU/L at some point during the period of the test.

Interpretation: In patients with active disease, glucose fails to suppress GH, instead there may be a paradoxical rise. Often there is evidence of decreased glucose tolerance. A paradoxical rise may also occur in renal failure and diabetes mellitus. Failure of suppression is sometimes seen in advanced liver disease, heroin addiction and anorexia nervosa.

Comments: This is a useful test for confirming suspected early acromegaly, or for establishing whether or not obvious acromegaly is still active. In burnt out acromegaly, the basal serum GH level returns gradually towards normal, although impaired glucose tolerance may persist.

EXERCISE STIMULATION TEST

Principle: Strenuous physical exercise causes stimulation of GH secretion in normal subjects.

Preparation: The patient should be fasting overnight and the test should be performed early in the morning (0800 hours).

Procedure: Basal blood specimen is obtained and the patient is subjected to rigorous exercise on a treadmill for 15-20 min. A blood specimen is taken 10 min after the cessation of exercise. GH estimation is done on both the samples.

Interpretation: Normally serum GH should rise to >20 mIU/L. The response is inadequate in GH deficiency.

L-DOPA STIMULATION TEST

Principle: L-Dopa stimulates growth hormone (GH) secretion from the anterior pituitary gland.

Preparation: The patient should be fasting overnight and test should preferably be carried out in the morning.

Procedure: L-Dopa is administered orally preferably with food and milk according to the following dosage schedule:

Patient >30 Kg: 500 mg
Patient between 15-30 Kg: 250 mg

Patient <15 Kg: 125 mg

Sampling: 5 ml venous blood is collected at 0 (basal), 60, 90, and 120 min after L-Dopa administration.

Interpretation: If GH level >20 mIU/L GH deficiency is unlikely.

GH level between 10-20 mIU/L is suggestive of partial GH deficiency

GH levels <10 mIU/L indicates GH deficiency.

Comments: In view of the imagined response seen in some normal subjects, the test is of greater value in excluding GH deficiency. An impaired response should be confirmed by other GH stimulatory tests. This test is the best alternative to the insulin stress test for hypothalamic pituitary (anterior) assessment in adults. Side effects of this test may include transient nausea and occasional vomiting.

INSULIN STRESS TEST

This test is used as the standard provocative stimulus for assessing reserve function of GH and ACTH and can also be used for PRL studies.

Principle: The stress of rapidly produced insulin induced hypoglycaemia of sufficient degree stimulates, via the hypothalamus, the release of growth hormone (GH) adrenocorticotrophic hormone (ACTH) and prolactin (PRL) from the anterior pituitary gland. Measurement of these hormones in blood is an estimation of functional pituitary reserve.

Caution: This test is potentially dangerous, especially in children, and is contraindicated in patients with epilepsy, ischaemic heart disease and primary adrenocortical insufficiency.

Preparation: Any replacement steroid therapy should be discontinued 12 h prior to the test (caution: this may be hazardous). Dopamine blocking drugs, which raise serum PRL levels, should not have been taken for at least 2 weeks prior to the test. The patient should be fasting overnight. At least 30 min should be allowed to elapse following insertion of an intravenous catheter before collecting the baseline blood samples. The test should be performed in the morning and under constant supervision.

Procedure: Soluble insulin (0.10-0.15 units/kg body weight) is the standard dose given intravenously 0.05 units/kg body weight is appropriate if marked hypopituitarism is suspected and 0.3 units/kg body weight if insulin resistance is anticipated, e.g., in acromegaly, Cushing's disease and obesity. If symptomatic hypoglycaemia has not occurred 45 min after the injection of insulin, a further dose of 50% of the amount given should be administered at this stage. Samples of venous blood (5 ml) are collected at 0, 20, 30, 45, 60, 90, and 120 min and divided between tubes containing heparin, plain glass tubes and glass tubes containing fluoride/oxalate for estimation of plasma ACTH (special collection), serum cortisol, GH and PRL and plasma glucose respectively. Following the test the patient should be given a carbohydrate-rich meal and observed carefully, especially for the next 2 h. Though not generally recommended, this test may be performed on out patients, in which case 5 mg prednisone should be given orally at the end of the procedure.

Caution: Great vigilance is required throughout the test for hypoglycaemia, which may occur as early as 15-30 min after administering insulin. If the symptoms are prolonged 20 ml of 50% glucose solution should be given intravenously. This will not invalidate the test.

Interpretation: It is necessary for the plasma glucose to fall to <2.2 mmol/L for this test to be valid. It should return to the reference range by 30 min. There should be a marked rise in the measured pituitary and target organ hormones with the different responses peaking at 20-90 min. The degree of the various responses varies widely. Impaired hormonal response to this may be overall or selective. High basal levels of serum GH, cortisol and PRL may indicate a stress reaction. High serum PRL levels alone may be due to a prolactinoma. **Hypopituitarism** is characterised by failure of all anterior pituitary hormones to rise. Isolated failure of serum GH levels to rise may be due to primary hypothyroidism. All the responses must be considered together and viewed carefully in light of the whole clinical context before any conclusions are drawn as to the assessment of hypothalamic anterior pituitary reserve function.

Comments: This test is uncomfortable for the patient. Measurements of plasma ACTH may well be omitted, as there are difficulties in performing this assay. Serum cortisol will suffice on the assumption that adrenocortical function is intact. Insulin is only one of several factors known to increase the GH release. Others include exercise, arginine, bovril, clonidine, and L-dopa.

POSTERIOR PITUITARY

Most common disorder involving posterior pituitary is diabetes insipidus, which results from deficient production of ADH, which is evaluated by following tests.

WATER DEPRIVATION TEST

Principle: In patients with polyuria, the response of both urine osmolality and output to water deprivation differentiates overhydration from diabetes insipidus, as long as osmotic diuresis and chronic renal failure have been excluded.

Indication: The test is useful for the assessment of patients with polyuria suspected of having water intoxication (including iatrogenic intoxication and psychogenic polydipsia) or diabetes insipidus of hypothalamic, posterior pituitary or nephrogenic origin. Diabetes mellitus, other causes of osmotic diuresis and renal failure must previously have been excluded.

Caution: Prerenal uraemia is a hazard in patients having renal impairment.

Preparation: The patient fasts overnight and during the procedure, but free access to fluids should be allowed prior to the commencement of test. The patient should rest in bed. Smoking is not permitted.

Procedure: The patient should pass urine in the early morning with suprapubic pressure in order to ensure complete emptying of the bladder. The urine is saved. Venous blood (5 ml) should be collected at approximately 9.00 am into a plain glass bottle. Urine aliquot is also collected at this time into a plain glass bottle. The patient now commences the phase of complete fluid deprivation and is weighed accurately at this point. Blood and urine sample are repeated later in the day and if necessary again the following day until the serum osmolality rises to >295 mmol/kg; however measurements need not normally continue for more than 48 h. The patient should be weighed again during, and at the end of the test. Patients with suspected psychogenic polydipsia should be observed closely throughout the test to prevent surreptitious water intake.

Sample handling: Blood and urine should be handled as for electrolyte assays.

Normal response: The serum osmolality should not rise to >295 mmol/kg at any time but the urine osmolality should rise rapidly towards 800 mmol/kg accompanied by a marked fall in volume. The serum sodium concentration should not rise to >144 mmol/L and the patient should not lose >3% body weight at maximum.

Interpretation:

1. A high baseline serum osmolality rising rapidly during the test, together with failure to develop an appropriate response in urine osmolality, and accompanied by persisting high urine volumes indicates diabetes insipidus of hypothalamic, posterior pituitary or nephrogenic origin.
2. Patients with hypothalamic diabetes insipidus (including those with neurosurgical damage particularly following removal of a craniopharyngioma) will, in addition, develop significant hypernatraemia and may characteristically show decreased or absent thirst.
3. Patients with diabetes insipidus of posterior pituitary origin may exhibit rapid loss of up to 3% of body weight and will become unwell at which point the test must be discontinued; patients will also exhibit very severe thirst.
4. Patients with iatrogenic water intoxication (e.g., inappropriate intravenous fluid therapy) will show a normal but delayed response.
5. Patients with psychogenic polydipsia will also show a normal response, however, due to the chronic nature of the condition there may be impairment of ability to concentrate the urine, resulting in a less than optimal rise in urine osmolality.
6. Patient with polyuria due to chronic renal failure would display high serum osmolality on account of mild dehydration in addition to the elevated serum urea; the serum osmolality would continue to rise further during the test with little change in urine osmolality.
7. However, water deprivation should not be performed in patients with known renal failure and such patients should be excluded from this procedure.

DESMOPRESSIN ACETATE RESPONSE TEST

The test is used to confirm the diagnosis of nephrogenic/neurogenic diabetes insipidus.

Principle: Exogenously administered desmopressin acetate (1-deamino-8-D-arginine desmopressin DDAVP) fails to lessen the diuresis in either congenital or acquired nephrogenic diabetes insipidus. These disorders are characterised by renal tubular end organ resistance to vasopressin thus differ from hypothalamic/pituitary diabetes insipidus, in which conditions a positive response occurs in this test.

Preparation: For several hours prior to the test, free access to fluids is encouraged. Smoking is not permitted.

Caution: This test could precipitate water intoxication in association with marked but temporary urine suppression.

Procedure: In the morning the bladder is emptied completely, the urine being saved in a

plain glass bottle. Venous blood (5 ml) is collected into a plain glass bottle. The aqueous preparation of DDAVP (4 µg) is administered intramuscularly, and further samples of venous blood and urine are collected at hourly intervals for a period of 4 h.

Sample handling: This is as for estimation of sodium potassium and osmolality in serum, and osmolality in urine. Urine volumes are also recorded.

Interpretation: There should be marked fall in urine volume and a marked rise in osmolality. The serum osmolality should be reduced to near the lower limit of the reference range (280 mmol/kg) and although the serum sodium concentration may also fall slightly, it should nevertheless, remain within the reference range as also should serum potassium. Patient with nephrogenic diabetes will fail to respond adequately to DDAVP, maintaining high urine output of low osmolality, with serum osmolality above the upper limit of the reference range (290 mmol/kg). The serum sodium concentration will be near to or above the upper limit of the reference range. Patients with pituitary or hypothalamic diabetes insipidus or psychogenic polydipsia respond to DDAVP administration by showing a fall in urine volume and a rise in osmolality, though in the latter disorder the response may take several days to become fully manifested. Indeed patients with a marked and prolonged diuresis from any cause may respond poorly to DDAVP initially.

Comments: It is not appropriate to perform this test in patients with polyuria due to chronic renal failure or osmotic diuresis. Serum potassium measurement are indicated when there is prolonged urinary suppression following DDAVP, though this is less likely to occur than was the case with the earlier long acting oily preparations of vasopressin. Interpretation of this test should be considered in conjunction with the water deprivation test.

THYROID GLAND

The thyroid gland consists of two lobes connected by a thin isthmus. Each lobe is located on either side of trachea. The structural unit of gland is a follicle, which consists of an outer layer of epithelial cells and filled with colloid. The colloid is mainly composed of thyroglobulin. The thyroid gland secretes thyroxine (T4) and tritodothyronine (T3) that influence most of the metabolic processes of the body. The synthesis is accomplished under the influence of thyroid stimulating hormone (TSH) from iodide and tyrosine residues. The hormones are stored in the follicles and released into peripheral circulation when required. The thyroid gland also has parafollicular or C cells, which produce calcitonin. The thyroid function is evaluated by estimating the levels of T3, T4 and TSH in blood. In few situations evaluation of dynamic function of thyroid gland needs to be carried out with TRH stimulation test.

ADRENAL CORTEX

The adrenal glands are extraperitoneal structures situated at the upper poles of the kidney. Each gland consists of an outer cortex, which synthesis steroids, and an inner medulla, which produces catecholamines. The hormones (steroids) produced by the adrenal cortex, consists of three distinct groups: the mineralocorticoids (e.g., aldosterone), the glucocorticoids (e.g., cortisol), and the adrenal androgens. The precursor compound in the synthesis of all adrenal steroids is acetate or cholesterol. The adrenal cortical function is evaluated by estimating the levels of hormones secreted by adrenal cortex. Evaluation by dynamic function tests is required in many situations.

SHORT ACTH STIMULATION TEST

This test is of value in patients with suspected primary adrenocortical insufficiency, e.g., Addison's disease and also during the later stages of withdrawal and following total cessation of previous long term high dose glucocorticoid drug therapy including topical preparation.

Principle: Synacthen is a synthetic preparation comprising the first 24 amino acids of ACTH. It stimulates the normal adrenal cortex to produce cortisol. Failure to respond indicates impaired adrenocortical function.

Preparation: This test can be used either as an in-patient or out patient screening procedure. The patient is placed in a reclining position to rest for 30 min prior to the test. Smoking is not permitted. Pharmacological doses of glucocorticoid should not have been administered for the previous 12 h.

Caution: Withdrawal of glucocorticoid may be dangerous.

Procedure: This test is best performed early in the morning. Baseline venous blood (5 ml) is collected into a plain glass bottle. Synacthen (250 µg) is administered intramuscularly or intravenously and 30 min later a further blood sample is collected for serum cortisol.

Normal response: The baseline serum cortisol level should be >140 nmol/L. This should rise at

30 min to >550 nmol/L, with the rise being >200 nmol/L irrespective of the initial level.

Interpretation: Failure to meet the normal criteria indicates adrenocortical insufficiency due to any cause. Low normal levels and responses are an indication for further investigation using the depot forms of Synacthen i.e., the five hour synacthen stimulation test. A clearly normal response excludes primary and secondary adrenocortical insufficiency and indicates that further tests are not required.

Comments: This investigation is frequently done being a safe, useful and practical screening test. Allergic reactions to Synacthen are a possibility, but rarely occur. It is often used repeatedly in order to assess adrenocortical function during the later stages of slow withdrawal of prolonged, high dose glucocorticoid therapy. It may also be used to confirm a previously made diagnosis of Addison's disease in patients receiving replacement therapy.

PROLONGED ACTH STIMULATION TEST

This test is indicated for confirming clinically suspected primary adrenocortical insufficiency in patients in whom there is a doubtful response in the short synacthen stimulation test.

Principle: Synacthen is a synthetic preparation comprising the first 24 amino acids of ACTH. It stimulates the normal adrenal cortex to produce cortisol. Failure to respond indicates impaired adrenocortical function.

Preparation: This test can be used as an in-patient or out patient procedure. The patient is placed in a reclining position to rest for 30 min prior to the test. Smoking is not permitted. Pharmacological doses of glucocorticoid should have been avoided for the previous 12 h.

Caution: Withdrawal of glucocorticoid may be dangerous.

Procedure: This test is best commenced early in morning. Baseline venous blood (5 ml) is collected into a plain glass bottle for serum cortisol and a further 2 ml may be collected at the same time into a polythene bottle containing heparin, pre-cooled on ice for plasma ACTH estimation. Synacthen depot 1 mg is injected intramuscularly. Venous blood (5 ml) is collected 1 h and 5 h later for serum cortisol estimation.

Sample handling: This is as for serum cortisol and plasma ACTH estimation. The sample for ACTH estimation should be processed immediately.

Normal response: The baseline serum cortisol should be >140 nmol/L. This should rise at 1 h to between 600 and 1250 nmol/L and at 5 h to between 1000 and 1800 nmol/L. The baseline plasma ACTH should lie within the reference range (10-80 ng/L).

Interpretation: A normal response excludes primary adrenocortical hypofunction, but does not exclude hypofunction secondary to pituitary disease or prolonged excessive glucocorticoid therapy. An impaired response suggests the prolonged synacthen stimulation test to differentiate primary or secondary adrenocortical insufficiency. A normal baseline plasma ACTH level excludes primary adrenocortical insufficiency.

LOW DOSE DEXAMETHASONE SUPPRESSION TEST

This test is indicated in patients with affective disorders in whom there is clinical suspicion of endogenous depression.

Principle: The normal response of serum cortisol suppression following a standard dose of dexamethasone given orally is absent in approximately 50 % of patients suffering from affective disorders with a significant element of endogenous depression, due to failure of negative feed back to suppress the limbic system.

Preparation: There should have been no treatment with glucocorticoid drugs (including topical preparations) for several weeks. Mineralocorticoids do not interfere with this test. The test may be performed on in patients or out patients.

Procedure: Venous blood (5 ml) is taken into plain glass bottle at 9 am and 4 pm on the first day. At 11 pm on the same evening, dexamethasone (1 mg) is given orally. A further blood sample is taken at 9 am and 4 pm on the following day.

Sample handling: This is as for serum cortisol estimation.

Normal response: The baseline 9 am serum cortisol value on the first day should be within the reference range (140-640 nmol/L). There is marked suppression of the 9 am serum cortisol value on the second day (i.e., 10 h after the dexamethasone dose) this remains low at 4 pm (<180 nmol/L) persisting for a total period of 24 h.

Interpretation: A significant proportion of patients with depression (in whom there is loss of the normal diurnal variation in serum cortisol levels) show early escape from the suppression of serum cortisol normally seen at 4 pm on the second day as evidenced by a concentration of >180 nmol/L or >50 % of the value found at 4 pm on the first day. However, many patients with depression fail to show this escape by exhibiting

low serum cortisol concentrations. It may also be found in individuals with organic hypofunction of the adrenal cortex but these patients would show low levels in baseline sample too. Marked hepatic microsomal P-450 enzyme induction by drugs leads to dexamethasone being eliminated rapidly, thereby causing inadequate suppression of serum cortisol. Some patients with other disorders, including anorexia nervosa without obvious depression, weight loss and patients with dementia associated with enlarged cerebral ventricles show false positive responses, i.e., they, too display escape from suppression. About 20% of normal subject also show a positive response. The test is negative in patients with pure anxiety states and schizophrenia, but it must be remembered that these disorders may be associated with an element of depression in which case the test could be positive. A repeat test following treatment for the depression, which remains positive, suggests a poor prognosis.

Comments: The 9 am blood sample taken on the second day showing a low serum cortisol concentration as compared with a normal baseline value on the first day confirms compliance. This knowledge is important when assessing depressed patients exhibiting early escape i.e., by finding normal serum cortisol levels at 4 pm on the second day. Some authorities recommend measurement of serum dexamethasone concentrations as a further check on compliance in addition to assaying serum cortisol.

HIGH DOSE DEXAMETHASONE SUPPRESSION TEST

This test is useful to determine the cause of Cushing's syndrome and in differentiation of pituitary dependent Cushing's disease from the other causes of Cushing's syndrome including ectopic ACTH production by tumours, adrenocortical adenomas and adrenocortical carcinomas. The latter 3 disorders do not usually show suppression.

Principle: A high dose of dexamethasone administered over a short period of time differentiates pituitary-dependent Cushing's disease from Cushing's syndrome of other aetiology by causing suppression of plasma ACTH serum cortisol in the former.

Patient preparation: The patient should be admitted to hospital. There should have been no treatment with glucocorticoids (including topical preparations) for several weeks; mineralocorticoids do not interfere with this test. The test is contraindicated in cardiac failure.

Procedure: Dexamethasone (2 mg) is administered orally 6 hourly over a period of 48 h, i.e., a total of 16 mg is given. Venous blood is collected for serum cortisol and plasma ACTH estimation immediately before starting the test and 6 h after the last dose.

Sample handing: This is as for estimation of serum cortisol and plasma ACTH. The sample for ACTH estimation should be processed immediately.

Normal response: There is marked suppression of serum cortisol to <50% of the baseline level 6 h after the last dose of dexamethasone.

Interpretation: Suppression of serum cortisol to <50 % of the baseline level in patients with Cushing's syndrome points to a pituitary dependent aetiology. Failure of suppression is a feature of both adrenocortical tumours (adenoma and carcinoma) and ectopic ACTH producing tumours Failure of suppression may occur particularly in some patients with pituitary disease. Measurement of baseline plasma ACTH discriminates between adrenocortical tumours in which it is low. An extremely high serum cortisol level favours the diagnosis of adrenocortical carcinoma or ectopic ACTH producing tumours. A paradoxical response (i.e., a rise of serum cortisol) should alert one to the possibility of cyclical Cushing's syndrome.

Comments: This test is time consuming and not without adverse clinical effects, particularly in patients with incipient cardiac failure, hypertension and peptic ulcer. It is currently less frequently performed. Suppression of a high baseline plasma ACTH following dexamethasone administration occurs in patients with pituitary dependent Cushing's disease, but adds nothing to the information gained from confirming cortisol suppression alone. Dexamethasone does not interfere with the measurement of serum cortisol.

ADRENAL MEDULLA

It secretes catecholamines (epinephrine and norepinephrine). These hormones are catabolised into metanephrine and normetanephrine and finally into venillyl mandelic acid (VMA). Inappropriate catecholamine overproduction is usually due to pheochromocytoma. Measurement of plasma catecholamines and urinary VMA are helpful in the diagnosis.

TESTES

The mature testis comprises seminiferous tubules that produce spermatozoa and Leydig cells that synthesise male sex hormones

(testosterone and DHT). Anterior pituitary hormones (FSH and LH) under the influence of Gonadotrophic releasing hormones (GnRH) of hypothalamus control the testicular activity. Testosterone in the circulation binds mainly with sex hormone binding globulin (SHBG). Its free form traverses the cell membrane of target tissue where it is converted to dihydrotestosterone (DHT). The circulating androgens are metabolised in liver and excreted through urine as 17-ketosteroids. The biological effects of androgens are genital differentiation, development of secondary sex character, skeletal muscle growth, deepening of voice, and social behaviour. Testosterone can be measured in blood. Its metabolic product, 17-ketosteroid can be measured in urine. However, in assessing testicular function it is important to know whether there is a residual gonadal tissue or not.

HCG STIMULATION TEST

Principle: Human chorionic gonadotropin (HCG) stimulates secretion of testosterone from Leydig cells of testes. The response to administration of HCG is proportional to residual functional testicular tissue.
Indication: Differential diagnosis of male hypogonadism.
Procedure: HCG injection (Profasi) 2000 units given intramuscularly on day 0 and 2. Serum testosterone is measured on day 0, 2 and 4.
Interpretation: In normal persons serum testosterone levels rise to outside the reference range. In hypogonadism, a failure of testosterone to rise after HCG stimulation suggests the absence of functioning testicular tissue. Conversely a rise shows that testicular tissue is present which may be intra-abdominal, if none is palpable in the scrotum.

OVARIES

The mature ovary is hormonally dedicated to the development maturation, release, and fertilization of an ovum every month during reproductive life. These repeated cyclical changes in ovary and uterine endometrium constitute the menstrual cycle. It is controlled by pituitary hormones (FSH and LH) that are under the influence of GnRH of hypothalamus. The oestrogen is synthesised mainly in the theca cells of ovarian follicle and secreted into the circulation. It is mainly bound to SHBG, the free form acts on target tissues. The biological effects of oestrogen are genital differentiation, development of female secondary sex characters, and social behaviour. Another hormone (progesterone) is mainly synthesized in the granulosa cells of follicles after ovulation and has its effect on endometrium during luteal phase. These hormones are metabolised in liver and excreted through kidney. Measurements of female sex hormones along with pituitary gonadotrophic hormones at various phases of menstrual cycle provide information regarding ovulation (fertility). Following test is commonly performed.

SCREENING TEST FOR OVULATION

Indication: To confirm ovulation in a woman with regular periods presenting with infertility.
Background: LH and FSH rise for approximately 48 hours (surge) at the onset of the ovulatory phase of the menstrual cycle. Progesterone production rises to a maximum during the luteal phase.
Preparation: Confirm menstruation and exclude other causes of infertility e.g., hyperprolactinaemia, chromosomal problems, thyroid dysfunction.
Procedure: Arrange for blood to be a taken every 2 days from day 18 to day 24 for LH, FSH, progesterone, estradiol (E2), looking for rise to values: LH >20 IU/l, FSH >10 IU/l, E2 >450 pmol/L, progesterone >30 nmol/L between days 20 and 24 is indicating an adequate luteal phase (production of progesterone by granulosa cell). It should be undertaken for at least 2 cycles.
Interpretation: Evidence of ovulation and adequate luteal phase should prompt further investigation of causes of infertility unrelated to ovulation or menstrual cycle (husband's sperm count, fallopian tube defects etc).

CLOMIPHENE STIMULATION TEST

This test is indicated for differentiating delayed puberty from isolated hypogonadotrophic hypogonadism. It may also be used in conjunction with the gonadotropin-releasing hormone (GnRH) stimulation test for differentiating hypothalamic from pituitary lesions.
Principle: Clomiphene citrate is a non steroidal compound which blocks hypothalamic steroid receptors, thereby preventing natural gonadal steroids from producing a negative feedback to the hypothalamus, there is, as a result release of gonadotrophin-releasing hormone which causes increased secretion of leutinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gland.
Preparation: No special reparation is required, but the patient should not have been receiving steroid compounds. The test may be performed

on in patients or out patients.

Procedure: Adult females should receive clomiphene citrate (100 mg) daily orally for 5 days, commencing on the 5th day of the menstrual cycle. For adult males, clomiphene citrate (100 mg) is administered twice daily by mouth for 10 days. Venous blood is collected before commencing

Normal response: In adult males serum testosterone and FSH should rise by >50% and LH by >75% from baseline levels within the reference range. In adult females, serum LH and FSH should at least double, rising from baseline levels within the reference range.

Interpretation: In males before puberty and patients with hypothalamic or pituitary disease and those with isolated LH deficiency show reduced or absent responses of serum LH, FSH and testosterone from low baseline levels. Further elevation of serum LH occurs in patients with primary disease exhibiting a high baseline level of serum LH with reduced serum testosterone.

54. CLINICAL TOXICOLOGY

Clinical toxicology may be defined as the analysis of drugs, heavy metals and other chemical agents in body fluids for purpose of patient care. Clinical laboratories need to provide tests for the cases in which a poisoning or drugs overdose is suspected. Depending on the situation, complete blood count, and urinalysis, blood gases, electrolytes, glucose, urea, LFTs and osmolality are general tests that should be obtained for any potentially toxic patient. These tests not only assess metabolic and organ functions but also allow the determination of the anion and osmolal gap, which help in diagnosis, and management of poisoned patient. In clinical toxicology, it is neither possible nor necessary to test for all of the hundreds of drugs or potentially toxic chemicals. Thus, the scope of toxicology testing will depend on the pattern of local drug use and on the available resources. Knowledge of the specific drug/toxin present in blood and body is important for the diagnosis and treatment of the specific drug poisoning in clinical practice. Patient's history is very helpful but is not always accurate, especially when the patient has taken an illicit substance. Signs and symptoms at presentation may provide clues to the nature of poisoning. Patients may present with or without symptoms, which may be useful to determine the nature of substances actually present. Toxicology testing for a substance for which a specific antidote is available is useful. Quantification before and after therapy may be helpful to determine the adequacy of treatment. Toxicology testing may identify multiple drugs. **Negative** results may lead the clinician to consider other aetiologies. Thus toxicology testing is beneficial and appropriate for patient care. Classification and clinical presentation of poisons is as under:

GROUP-1 POISONS (GASES)

Symptoms: Apnoea, asphyxia, dyspnoea, vomiting; pink or red skin colour (carbon monoxide or cyanide)
Onset of symptoms: Very rapid onset of illness or death
Scene: Hospitals, industrial sites, laboratories, mines, bathrooms, boats, caravans, cars, fires, kitchens, geysers, portable heaters etc.
Occupation: Chemical industry, electroplating, furnaces, glue factories, industrial tank cleaning, metal treatment, mines, photography, sewers, tanneries.

GROUP 2 POISONS (VOLATILE COMPOUNDS)

Symptoms: Drunken behaviour (drowsiness, ataxia, speech and vision disturbance), jaundice (aniline, nitrobenzene), tremors, vomiting, and abdominal pain (especially with phenols).
Onset of symptoms: Rapid onset of illness or death when inhaled.
Scene: Domestic locations; hospitals and research laboratories; industrial locations, presence of liquor, methylated or surgical spirit; glues, anti-freeze or other domestic products.
Occupation: Degreasing plants, dry cleaners, printing; manufacturer of adhesives, dyes, paints, petroleum products, plastics, polishes, perfumes and rubber

GROUP-3 POISONS (DRUGS SOLVENT SOLUBLE)

Symptoms: Effects are variable but the following information may be used as a guide:
1. Analgesics: Gastric irritation, haematuria, tinnitus, sweating, comma, convulsions.
2. Opiates and synthetic narcotics: Contracted pupils, muscle twitching, slow respiration, hypertension and coma.
3. Sedatives and hypnotic: Ataxia, slurred speech, drowsiness, stupor and coma.
4. Stimulants and antidepressants: Dilated pupils, dry mouth, headache, tachycardia, tremors and convulsions

Onset of symptoms: Relatively slow unless injected (2-48 h)
Scene: Illegal lodgings, colleges, clubs, mental homes.

GROUP-4 POISONS (METALS)

Symptoms: Anaemia, cramps, diarrhoea, gastric pain, hair loss (thallium and selenium), jaundice, metallic taste, paralysis, peripheral neuritis, salivation, urine retention, vomiting, and weight loss
Onset of symptoms: Death may occur within 24 hours but more commonly after several days.
Scene: Industrial locations, laboratories
Occupation: Electroplating, smelting;

manufacture of agricultural chemicals, alloys, batteries, ceramics, glass and paint

GROUP-5 POISONS (PESTICIDES, SOLVENT SOLUBLE)

Symptoms: Principal features are vomiting, diarrhoea and convulsions. The following additional symptoms may be used as a guide:
Chlorinated hydrocarbons: Dizziness, headache, muscular weakness, tremors
Chlorinated phenoxyacetic acids: Burning sensation, low blood pressure
Organophosphates: Salivation, Contracted pupils, lacrimation, urination, sweating, dyspnoea, anoxia, cyanosis
Phenols and cresols: Fever, thirst, sweating, anoxia, haematuria, jaundice
Onset of symptoms: Rapid (within 30 min) if product contains a petroleum solvent or is inhaled. Otherwise, slow (1-6 h)
Occupation: Manufacture of agricultural chemicals, farm workers, gardeners, pesticide officers, food processing factories, domestic premises

GROUP-6 POISONS (ANIONS)

Symptoms: Vomiting, diarrhoea, abdominal pain, cyanosis (methaemoglobin formed with oxidising agents), stained skin and mucous (permanganate, oxalate, iodide and bleaching agents)
Onset of symptoms: usually within one hour, death may occur within several hours
Scene: Agricultural sites (nitrate, chlorate, fluoride, and fluoroacetate)
Industrial sites (nitrite, oxalate and sulphite)
Domestic sites, drain and lavatory cleaners (Hypochlorite and persulphate), weed killers (chlorate), insect powders fluoride) laboratories
Occupation: Sewer workers, rat catchers (fluoroacetate), factory workers

SCREENING OF DRUGS/TOXINS

Screening procedures are designed for the relatively rapid and generally qualitative detection of drugs/toxin. Basic clinical toxicological laboratories provide limited toxicology services in a useful time frame for patient care with photometric, immunoassay and chromatography.

ETHYL ALCOHOL AND VOLATILE COMPOUNDS

Ethanol is frequently abused chemical substance and measured in the toxicology laboratory. The principal pharmacological action of ethanol is CNS depression, euphoria, disorientation, incoordination, coma and death. Ethanol is metabolised in liver by alcohol dehydrogenase to acetaldehyde, which is subsequently oxidised to acetic acid, by aldehyde dehydrogenase. The intoxicating effects of ethanol on the brain are concentration related. Ethanol equilibrates between the brain compartment and blood, breath, serum, or plasma in the post-absorptive state because of its lipid solubility. The elimination rate is influenced by drinking habit and varies from 15 to 30 mg/dl/h.
Specimen: Blood, serum, urine and saliva are all appropriate specimen for this test. The site of venepuncture should be cleaned with alcohol free disinfectants such as aqueous zephiran (benzalkonium chloride), methiolate or other suitable disinfectant. Sodium fluoride is the best preservative and anticoagulant but citrate, oxalate and heparin can also be used as anticoagulants. All specimens must be kept capped to avoid evaporative loss to the atmosphere. Blood may be stored in refrigerator with properly sealed until they are analysed. Liquid paraffin prevents evaporation of ethyl alcohol and it may be added to cover the surface layer of blood sample. Urine ethanol concentrations are useful in determining alcohol abuse in person who has taken ethanol some time ago. For necropsy cases where putrefaction has begun, specimens of cerebrospinal fluid and aqueous humour from the eye should be taken. Whole blood ethanol measurements are more appropriately performed by gas chromatography (GC).
Plasma Osmolality and Serum Electrolytes: Measure the osmolality, preferably by freezing point depression. Calculate the osmolar gap and the anion gap. An osmolar gap of greater than 10 mosmol/kg suggests the presence of ethanol, isopropanol, methanol or ethanediol (ethylene glycol). The presence (or subsequent development) of a metabolic acidosis and an increased anion gap suggests methanol or ethanediol intoxication, although ethanol should first be excluded.
Ethanol Assay: Gas chromatography is the method of choice and offers a rapid means of identifying and measuring ethanol, methanol and isopropanol. Commercial kits for ethanol determination are available based on the enzyme alcohol dehydrogenase (ADH). These systems cross react with isopropanol, but show little cross reactivity with methanol or ethanediol.
Principle: Ethanol is measured by oxidation to acetaldehyde with NAD by alcohol dehydrogenase. Formation of NADH, measured

at 340 nm, is proportional to the amount of ethanol in the specimen. Reagent kits for use with manual spectrophotometer or automated analyser are available from several manufacturers such as Sigma and Abbott TDx. Perform the test according to manufacturers' instruction. **Alternative method** can be used if photometer does not have 340 nm filter:

$$\text{Ethanol} + O_2 \xrightarrow{\text{Alcohol Oxidase}} \text{Acetaldehyde} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + 4-\text{aminoperoxidase} \xrightarrow{\text{Antipyrine}} \text{Quinoreimine} + 4H_2O$$

Alcohol and Volatile Compounds:

Procedure 1: Dissolve the 0.5 ml sample in 0.5 ml 2M hydrochloric acid and add a few crystals of potassium dichromate with shaking.

Result

Immediate brown or green changing to brown	Aminophenol or a phenol group
YELLOW (→ BROWN)	Phenol (2 Min)
GREEN (→ BROWN)	Adrenaline, Dopamine, hexoprenaline, isoetharine, isoprenaline, levodopa, methytdopa, methyldopate, noradrenaline, rimiterot
Blue→green	Aniline (2 min)
BROWN	Benscrazide, o-cresol (30 s), ni-cresol (2 min), orciprenalin
(→ Red-brown on warming)	Terbutaline (slow)

Procedure 2: Add 1-2 drops of sample to 1 ml water followed by 1 ml a saturated potassium dichromate in 50% v/v sulphuric acid.

Result: Acetaldehyde, ethanol, methanol, and propanol give green colour.

PHOTOMETRIC SPOT TESTS

Chemical spot tests are rapid, sensitive, easily performed procedures that provide presumptive evidence of tested drugs. These tests may require some familiarity and testing of the drug with more specific method. The following drugs or drug class can be identified by spot colour tests in serum/urine:

Specimen Collection:

1. **Gastric Contents**: Vomit or aspirated first portion of stomach washings (50 ml) is sufficient. Note the smell, colour and general appearance and pH. Characteristic smells such as those due to phenolic disinfectants, camphor, methyl salicylate, cyanide, ethanol and other organic solvents should be noted. Tablets or capsules may be present and colours may derive from these. A green or blue colour suggests the presence of iron salts. A high *p*H indicates ingestion of alkalis. Remove any solid material by centrifugation and conserve it. Carry out tests on the supernatant.
2. **Blood**: Collect 10 ml heparinised blood and same amount of serum. Note the colour of the blood sample. A cherry red colour suggests carbon monoxide or cyanide poisoning. Confirmatory tests are given in the section on quantitative assays. A chocolate brown colour may indicate methaemoglobinaemia following poisoning with chlorates, nitrates, nitrites or other oxidising agents.
3. **Urine**: Obtain 50 ml of the first sample voided after admission and preferably before any drugs are administered in treatment, which might interfere with some of the tests.
4. **Others**: Any materials (tablets, powders, liquids, bottles, syringes) found with the patient should be retained for subsequent examination.

PARACETAMOL (ACETAMINOPHEN)

Principle: Acetaminophen is hydrolysed to *p*-aminophenol, which reacts with o-cresol and ammonium hydroxide to form an indophenol blue chromogen. Acetaminophen is a commonly used analgesic and antipyretic agent when taken in overdose may cause serious hepatotoxicity. Applicable to serum, urine, stomach contents and scene residues.

Reagents

1. Concentrated HCl (relative density 1.18).
2. o-cresol solution (10 g/L): Dissolve 10 ml o-cresol and make to 1 L with distilled water.
3. Aqueous ammonium hydroxide solution (4 mol/L): Dilute 284 ml concentrated ammonium hydroxide to 1 L with water.

Procedure

1. Mix 1 ml specimen and 1 ml concentrated HCL, boil for 10 min.
2. Cool and add 10 ml o-cresol solution to 0.2 ml of the above hydrolysate.
3. Add 2 ml ammonium hydroxide solution and mix for 10 seconds.

Results: A strong royal blue colour developing immediately indicates the presence of paracetamol. This test is very sensitive and can detect the drug after 24-48 hours.

Quantitative Assay

Kits based on homogeneous immunoassay or the enzymatic cleavage of paracetamol to *p*-aminophenol with subsequent colorimetric assay offer greater sensitivity. Applicable to plasma or serum.

Reagents

1. Aqueous trichloracetic acid (100 g/L)
2. Aqueous HCl (6 mol/L)
3. Aqueous sodium nitrite solution (100 g/L, freshly prepared).

4. Aqueous ammonium sulphate solution (150 g/L).
5. Aqueous sodium hydroxide solution (6 mol/L)

Standards: Prepare solutions containing paracetamol at concentrations of 0,50, 100, 200 and 400 mg/L in blank plasma. These solutions are unstable even at 4°C and must be prepared weekly or stored at -20°C.

Procedure

1. Add 2 ml trichloracetic acid to 1 ml sample or standard. Mix and centrifuge for 5 min.
2. In a separate tube add 1 ml of HCl to 2 ml sodium nitrite solution and mix. Brown nitrogen dioxide fumes may evolve.
3. Add 2 ml supernatant from step 1 to the mixture obtained in step 2, mix, and allow standing for 2-3 min at room temperature.
4. Add 2 ml ammonium sulphate solution drop by drop to remove excess nitrous acid.
5. Add 2 ml sodium hydroxide solution and measure the absorbance at 450 nm against plasma blank.

Result: Calculate plasma pracetamol concentration from standard curve. Paracetamol metabolites do not interfere, but the method is only useful within 4-24 h of ingestion and the limit of sensitivity (normally 50 mg/L) may be 100 mg/L or more with uraemic sera.

SALICYLATES

Applicable to urine, stomach contents and scene residues

Reagent: Trinder's reagent: Dissolve 4 g mercuric chloride in 85 ml water with 12 ml HCl (1 mol/L) and 4 g hydrated ferric nitrate. Dilute to 100 ml with water.

Method: Pipette 100 µl serum or urine to respective wells of a white porcelain spot. Add 100 µl Trinder's reagent to each well and swirl to mix.

Result: A strong violet colour indicates the presence of salicylates. Azide preservatives react strongly in this test, and urine specimens containing high concentrations of ketones bodies can give weak **false positive** result. This test is sensitive and will detect therapeutic dosage of salicylic acid acetylsalicylic acid, 4-aminosalicylic acid, methyl salicylate and salicylamide.

Quantitative Assay: Applicable to plasma or serum (1 ml)

Reagents

1. Trinder's reagent (see above)
2. Standards: Salicylic acid at concentrations of 0, 200, 400 and 800 mg/L.

Procedure

1. Add 5 ml Trinder's reagent to 1 ml sample or standard.
2. Mix for 30 seconds and centrifuge for 5 min.
3. Measure absorbance of supernatant at 540 nm against a sample blank.

Result: Calculate plasma salicylate concentration from the standard curve. Some salicylate metabolites interfere, but plasma concentrations of these compounds are usually low. Oxalates from fluoride/oxalate blood tubes interfere in this test.

PHENOTHIAZINES

It constitutes a large group of widely prescribed drugs with antipsychotic pharmacological action and can cause life-threatening intoxication with over dosage (coma and respiratory depression).

Reagents: FPN reagent: Mix 5 ml ferric chloride (50 g/L), 45 ml perchloric acid (200 ml/L), and 50 ml nitric acid (500 ml/L).

Procedure: Place 200 µl unknown and control urine samples into respective wells of a white porcelain spot test plate. Add 200 µl FPN reagent and rotate the plate to mix.

Result: Pink, red, purple, or blue colour forming immediately suggests the presence of phenothiazines. Tricyclic antidepressants may also react to give green or blue colours. The presence of phenylketonuria or liver impairment can give **false positive** reactions

TRICYCLIC ANTIDEPRESSANT

Tricyclic antidepressant drugs (Imipramine, Desipramine, and Trimipramine) are widely prescribed for the treatment of depression and may produce seizures, coma and cardiotoxic effects including arrhythmias, hypotension, and pulmonary oedema in over dosage.

Reagents: Forrest's reagent: Mix equal volumes of sulphuric acid (300 ml/L), perchloric acid (200 ml/L), nitric acid (500 ml/L), and potassium dichromate (200 mg/dL).

Procedure: Add 0.5 ml urine and 0.5 ml Forrest's reagents in a tube.

Result: Formation of a green, blue-green, or blue colour suggests the presence of imipramine, desipramine, or trimipramine. Phenothiazines may also give a positive result but the test is more sensitive for imipramine.

CARBON MONOXIDE

Carbon monoxide is a colourless, odourless, tasteless gas that is a product of incomplete combustion and causes cellular hypoxia because of carboxyhaemoglobin. Accidental poisoning s not uncommon in winter due to improperly ventilated home heating. It may

remain undiagnosed due to lack of laboratory tests facility. The gas disappears from the blood with a half-life of four to five hours and in a much shorter time if oxygen is administered. An IL CO-oximeter can quantitate Carboxyhaemoglobin rapidly. Alternatively photometric reference standards are also available.

Spectrophotometric method: Oxyhaemoglobin and carboxyhaemoglobin have similar double bands in alkaline solution. The absorption maxima for oxyhaemoglobin are 576 to 578 and 540 to 542 nm for carboxyhaemoglobin. Deoxyhaemoglobin has a single broad band at 555 nm (see also DETECTION OF CARBOXYHAEMOGLOBIN on page 45). Measured at 541 and 555 nm, absorbance ratio A 541/ A555 is calculated and the concentration per carboxyhaemoglobin is determined from the calibration curve.

1. **Reagents**: Ammonium hydroxide (0.12 mol/L); Dilute 15.9 ml concentrated ammonium hydroxide to 1.L with deionised water.
2. Sodium hydrosulphide (sodium dithionite); 10 mg sodium dithionite into individual small test tubes. Stopper the test tubes or cover with liquid paraffin.

Procedure:

1. Add 100 µl of heparinised blood to 25 ml ammonium hydroxide. Mix the solution (haemolysate) and allow to stand for 2 min.
2. Transfer 3.0 ml ammonium hydroxide and 3.0 ml the haemolysate to separate 10 mm tube.
3. Add 10 mg sodium dithionite to all tubes, cover with parafilm and invert gently 10 times.
4. Exactly 5 min after the addition of sodium dithionite to the haemolysates, read absorbance at 541 and 555 nm against the ammonium hydroxide blank.
5. Calculate the ratio of the absorbance at 541 nm to that at 555 nm, as A541/A555 and determine the percent carboxyhaemoglobin from the calibration curve.

CYANIDE

Death usually ensues rapidly after exposure to cyanide, although a small proportion of patients reach hospital and respond to antidotes and supportive treatment.

Procedure: To 5 ml filtered sample add 1 ml 4 M sodium hydroxide solution followed by five drops of freshly prepared 10% (w/v) ferrous sulphate solution. Add sufficient hydrochloric acid to dissolve the brown precipitate of ferrous hydroxide.

Result: A Prussian blue colour indicates the presence of cyanide.

ORGANOPHOSPHATE COMPOUNDS

This test detects the inhibition of serum cholinesterase as denoted by the development of much lower colour intensity in the tube containing the test sample.

Procedure: Add 3 ml dithiobisnitrobenzoic acid solution and 0.1 ml 5% (w/v) acetylthiocholine iodide solution to each of two tubes. Add 20 µl normal serum to one tube and 20 µl test serum to the other. Allow to stand for two min. Measure serum cholinesterase activity by commercial Kit.

Phosphorus Test Method: To the sample add 0.5 ml nitric acid and 0.2 ml sulphuric acid, heat at 100°C in water bath for 30 min, cool and add 1 ml 10% solution of ammonium molybdate. Place in water bath at 100°C for 5 min. A blank solution should be treated at the same time. For some compounds, the reaction may occur after heating for short time.

Result: A bright yellow solution or precipitate indicates the presence of phosphorus and suggests an organophosphorus pesticide.

BARBITURATES

Blood barbiturate assays are only justified in emergency if the clinician is considering active treatment such as forced alkaline diuresis or haemodialysis. Homogenous immunoassay systems are ideal for emergency work. Alternatively, a non-selective system can be used, coupled with a differential TLC screen on urine or stomach contents.

IRON

The first blood sample should be taken before giving the antidote; subsequent samples taken to monitor the progress of treatment must be treated with dithionite before analysis. About 20 mg solid sodium dithionite is dissolved in 1 ml serum. Haemolysed serum must not be used. The spectrophotometric method is suitable for emergency assay. Commercial kits for serum iron produce rapid and reliable results.

Ferrous and Ferric Iron: Add three drops of 2 M HCl and one drop 1% (w/v) potassium ferricyanide solution to two drops of urine/stomach sample.

Result: Deep blue precipitate indicates ferrous/ferric iron.

HALOGENATED HYDROCARBONS

(CHLOROFORM, CARBON TETRACHLORIDE CHLORAL HYDRATE)

Reagents

Ether
Pyridine
NaOH (5 mol/L)
Procedure: Extract 1 ml blood with 2 ml ether. Take 1 ml of the ether layer in a test tube, add 1 ml pyridine and 1 ml NaOH. Place in boiling water bath for 1 min.
Result: A deep red colour developing in the pyridine layer indicates presence of chloroform, chloral hydrate, dichloralphenazone, chlorbutol and trichloroethylene. Carry out the procedure with 1 ml aqueous solution of trichloracetic acid (10 mg/L) as standard

OXIDISING AGENTS

The test detects hypochlorite (from domestic bleach), bromates, chlorates, dichromates, iodates, nitrates, nitrites and permanganates.
Procedure: Add two drops of filtered sample to 1 ml diphenylamine reagent.
Result: A deep blue colour appearing immediately indicates the presence of an oxidising agent.

PHENOLS

(CRESOLS, NAPHTHOLS, THYMOLS)
Reagents
Ether, Sodium nitrite ($NaNO_2$) fresh 0.14 molar in H_2SO_4, concentrated H_2SO_4 and NaOH pellets
Procedure: Extract 2 ml stomach contents with 10 ml ether, evaporate ether in a small white crucible at room temperature and add 1 drop freshly prepare $NaNO_2$, one drop water and 1 pellet of NaOH.
Result

Cresols:	Dark brown
Naphthol:	Green
Phenols:	Reddish green to blue
Thymols:	Green to purple

OXALIC ACID/OXALATES

Reagents
$KMnO_4$ (0.1 g/dl H_2O), concentrated HCl, saturated $CaCl_2$, and NH_4OH
Procedure: Adjust *p*H of specimen to 1.5 with concentrated HCl. Re-adjust *p*H of 5 ml aliquot to approximately 8 with NH_4OH and add saturated $CaCl_2$ solution drop wise.
Result: Formation of precipitate is indicative of oxalates. Add concentrated HCl drop wise if precipitate dissolves, presence of oxalate is the probably. For further confirmation, warm the mixture and add $KMnO_4$ solution drop wise. Disappearance of violet colour confirms the presence of oxalate.

HEAVY METALS

(ANTIMONY, ARSENIC, BISMUTH, MERCURY)
Reagents:
Copper wire 10 cm
HCl 2 M
Sand paper
Procedure:
1. Clean copper wire with sand paper and make a into a coil
2. Take 5-10 ml specimen and add equal volume of HCl.
3. Place the copper wire in this mixture.
4. Place in a boiling water bath for 10 min.
5. Examine the wire for any stain within on hour.

Results:

Antimony:	Blue or purple black
Arsenic:	Dull black
Bismuth:	Shiny black
Mercury:	Silver grey

Confirmatory tests are essential. The clinical features should suggest the type of metal involved and quantitative assays on blood or urine or both should be carried out, preferably by atomic absorption spectrophotometry.

IMMUNOASSAY FOR DRUGS

Different types of immunoassay like EMIT, FPIA and RIA screening methods have been designed for detection of drugs/ toxic compounds in urine and serum in emergency. These results may be sufficient for clinical purposes when interpreted with relevant clinical information. However, in the absence of clinical data or for forensic purposes they should be regarded as presumptive. It is advisable to verify or confirm the result by another assay. For assessing performance of these assays we recommend that two levels of control materials (one positive and one negative) be performed with the assays.. Controls should be selected with analyte concentrations close to the cut-off values to distinguish positive from negative results.

THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) is a versatile procedure that requires no instrument. Most of the basic, acid and neutral drugs can be detected from the specimen of the poisoned patient. Urine is specimen of choice. The TLC plates most commonly used in clinical toxicology laboratories are designed for rapid specimen application, solvent migration and detection. We can identify many acid, basic and neutral drugs.

Commercial Toxi-lab kits are available. See also THIN LAYER CHROMATOGRAPHY on page 40 for details.

Procedure: Divide a ready made TLC plate (silica gel G, 250 µm) into eight equal columns by scoring lines with a spatula and draw a horizontal line 10 cm from the origin. Apply 10 µl standards and the extract of urine in the sequence. Develop the plate in 100 ml of ethyl acetate; methanol; ammonia mixture (85:10:5) and dry thoroughly under a stream of cold air. Spray the plate with appropriate staining solution. Note that reference values are a useful guide only; authentic compounds must be analysed alongside the sample extracts to obtain more reliable evidence.

SECTION VIII - HISTOPATHOLOGY

55. SPECIMEN COLLECTION AND TRANSPORT

COLLECTION OF BIOPSY SPECIMENS

Fixative: Surgical specimens after removal should be placed in an adequate quantity of fixative (10% formal saline) as soon as possible. For optimal fixation a piece of tissue should be immersed in at least 10 times its own volume of fixative.

Containers: Jars or bottles with screw tops and of suitable capacity should be used. Large specimens should not be squeezed into a smaller container. This will result in inadequate fixation and will allow autolysis to proceed unchecked. The subsequent interpretation of microscopic appearances may thus be made difficult or impossible. If a specimen is too large to fit easily into the largest size of container it should be brought as such to the laboratory without delay in a bucket or other suitable container. Amputated limbs may be wrapped in a rubber sheet. Bulky solid specimens, e.g., large tumours, spleen, etc. should be bisected cleanly with a large sharp knife before being placed in fixative. Hollow viscera such as portions of stomach and intestine should be opened at both ends or cut open along their length (stomach should be opened along the greater curvature). If, for any reason, jars of fixative are not available, the specimen should be taken fresh to the laboratory or wrapped in moist cotton wool and put in the refrigerator overnight. The specimen should never be put into water or normal saline because this will hasten autolysis.

Rapid Frozen Section: If a rapid frozen section is required the laboratory staff and pathologist must be notified at least 1 hour before the time of the operation and preferably on the preceding day. Arrangements should also be made to notify the laboratory as soon as the patient is taken into the theatre for the start of the operation. The specimen must not be put into a fixative but be brought with the least possible delay to the laboratory. If after the start of the operation, the surgeon decides that a rapid frozen section is unnecessary the laboratory should be notified at once.

Request Forms: If more than one biopsy is taken from the same patient at the same time, a single request form will suffice for all those specimens relating to a single pathological lesion. If two or more apparently unrelated pathological lesions are biopsied from the same patient, separate forms should accompany each specimen. If previous specimens from the same patient have been sent to the laboratory, this fact should be stated on the form together with the approximate date of previous histological examination. If this was done in another laboratory, this fact should also be clearly stated. The medical officer in charge of the case must complete the forms. Apart from the usual particulars required on any request form the clinical data must include any specific information contributing or relating to the present illness. In surgical cases the following additional information is required:

1. Precise nature of the operation performed.
2. Whether the entire specimen or only a part of it is being sent to the laboratory.

Labelling of Specimen: It is the responsibility of the Medical Officer to see that the specimens are correctly labelled, including the name of the patient, ward, hospital and specimen; with date and time of obtaining the specimen. These particulars should tally with those stated in the accompanying request form.

Renal Biopsy: The specimen of renal biopsy for histopathological examination should be collected in 10% formal saline. The request form should contain all the relevant clinical information and results of laboratory investigations. The specimen of renal biopsy for immunofluorescence should be submitted fresh in normal saline.

Liver Biopsy: The liver biopsy specimen should be collected in 10% formalin/formal saline. Request form should mention the reports of LFTs and hepatitis markers tested besides the relevant clinical information. Liver biopsy specimen for the diagnosis of storage disorders should be collected in absolute alcohol.

Bone Specimen: Bone specimen should be collected in 10% formal saline. Information regarding age, sex of the patient, site of biopsy, clinical history and x-ray with radiologists opinion are required for reporting on bone specimens and should accompany the specimen.

Specimen for Immunohistochemistry and Tumour Markers: Specimens should be collected in 10% formal saline. If the case has been reported from AFIP initially, then AFIP

report No. is also required. If the case is reported by some other laboratory, then all the slides along with paraffin embedded blocks and the histopathology report are required for immunohistochemistry and tumour markers.

Specimen for Oestrogen and Progesterone Markers: These markers are carried out on paraffin blocks. Paraffin blocks along with previous slides and report are required if the case has been reported from some other laboratory.

Review Cases: In these cases full description of the gross specimen along with previous report, paraffin block, slides and clinical information are required.

COLLECTION OF CYTOLOGY SPECIMENS

Fixative: Two types of smears are used for cytological examination depending on the preferred method of staining. Usually wet-fixed smears are preferred to air-dried smears. Wet-fixed smears are prepared by immediately fixing the slide without allowing it to dry. The fixatives recommended are a mixture of equal parts of ether and 95% ethyl alcohol, formol alcohol or 95% ethyl alcohol alone. Not less than 15 min are required for adequate fixation though slides may remain in the fixative for 7-10 days without deterioration. Coplin jars made of glass or plastic are commonly used as containers for fixative. Papanicolaou and H&E stains are commonly used on wet-fixed smears. The air-dried smears are simply prepared by allowing these to dry naturally. These smears are stained with Giemsa stain, Leishman stain, etc. Smears must be labelled regarding type of preparation besides patient identification and fixation so that they can be appropriately stained.

Request Form: Request forms for cytological specimens must include identification data of the patient, type and site of specimen, clinical details and in case of slides, method of fixation used. In case of cervical smears date of last menstrual period, use of IUCD, hormone therapy, previous history, etc. must be mentioned on the request form.

Respiratory Tract: Sputum: A fresh early morning specimen produced by a deep cough should be collected and brought to the laboratory immediately without any fixation. If it is not possible to transport unfixed material to the laboratory, the sputum should be prefixed by asking the patient to expectorate into a wide-mouthed small jar half filled with 70% ethyl alcohol. In case of peripheral laboratories where cytology facilities are not available, fresh sputum should be examined grossly for tissue fragments and blood tinged areas. Smears from these areas and other randomly sampled areas should be prepared and fixed immediately (wet-fixed) in 95% ethyl alcohol. After fixation for 20 min these slides can be dried and transported to referral laboratory for reporting.

Bronchial Aspirates, Washings and Brushings: Aspirates and washings collected during bronchoscopy may be centrifuged and smears prepared from the cell button. Direct smears can also be made and fixed in 95% ethyl alcohol. Direct smears should be prepared from bronchial brushings and wet-fixed in 95% alcohol. Alternatively they can be processed like aspirates and washings.

Bronchoalveolar Lavage (BAL): BAL involves the infusion and re-aspiration of a sterile saline solution into the air passages. This fluid should be submitted as such immediately to the laboratory. If delay in transportation is expected, equal quantity of 95% ethyl alcohol should be added.

Urinary Tract: Urine: Freshly voided urine is the specimen of choice in male patients. The first morning specimen should be avoided as the urine has been collecting in the bladder overnight and the cells have usually degenerated. In female patients catheterised urine is the preferred specimen. Approximately 50-100 ml of urine should be collected in an equal amount of 50% ethyl alcohol. If possible the patient should be sent to the laboratory for collection of a fresh sample.

Washings and Brushings of Ureter and Renal Pelvis, Bladder Washings: All washings should be collected in an equal amount of alcohol for fixation. Brushings may also be added to alcohol for fixation. Alternatively direct smears may be prepared and wet fixed in an alcoholic fixative.

Pleural, Pericardial and Peritoneal Fluids: Fluid should be collected in a clean, dry container, which need not be sterile, and should be sent to the laboratory as soon as possible. If it is not possible to send the fluid immediately, it should be stored in a refrigerator at 4°C and not allowed to freeze. The specimen can be preserved at refrigerator temperature for several days. In case of small peripheral laboratories without cytology facilities, smears should be prepared after centrifugation. Both wet-fixed and air-dried smears of the sediment, labelled as such should be submitted to the referral laboratory.

CSF: Cerebrospinal fluid should be collected in a clean container and transported immediately to the laboratory for processing. If delay in transportation of more than a few hours is

expected equal amount of 50:50 alcohol ether mixture, or 95% ethyl alcohol may be added.

Alimentary Tract: Brush and wash samples may be collected from oesophagus, stomach, lower bowel and rectum. Brush smears should be prepared immediately by rolling on a clear glass slide. Five to six such smears may be wet fixed in alcohol for Papanicolaou staining. A few air-dried smears may also be prepared. These should be labelled as such and submitted for cytological examination. Wash samples from stomach must immediately be neutralised with N/10 sodium hydroxide (pH up to 6.0) and centrifuged rapidly in ice-cold siliconised tubes. Smears are prepared from the deposit in the same manner as mentioned above. Wash samples from lower bowel are also concentrated in the same way by centrifuging in ice-cold siliconised tubes and smears prepared.

Female Genital Tract: Cervical Smear: The aim of collecting a cervical smear is to obtain a representative specimen from the squamocolumnar junction (transformation zone), using an Ayre's spatula or similar device. Having exposed the cervix using a bivalve speculum a circumferential sample is obtained by rotating the spatula through 360°, thus obtaining cells from the entire junctional zone. If the smear does not show endocervical cells, an additional smear may later be obtained from the endocervical canal by using a cotton-tip applicator or endocervical cytobrush. Once the sample has been obtained it should be placed on a glass slide and spread smoothly down the length of the slide using a wooden spatula. The slide must be fixed immediately without allowing to, dry, by placing it in 95% alcohol for a minimum of 15 min or by using an aerosol spray fixative. After fixation the slide is allowed to dry and sent to the laboratory with a complete request form. Slide identification and labelling must be ensured at all times. In addition a sample may also be collected from the vaginal pool in the posterior fornix with the help of a pipette. Few drops of fluid are expressed onto a slide, spread and fixed in the same way as above. The advantage of this sample is that it contains cells from the entire female genital tract however the disadvantage is that the cells are dead and desquamated and may show degenerative changes, which are difficult to interpret.

Vaginal Smear: Specimens for hormonal evaluation are taken from the lateral vaginal wall. If this is not possible, posterior fornix pool specimen may be used. The specimens should be taken by lightly dipping the applicator in the secretions avoiding forceful scraping. The smears are prepared, wet fixed immediately in the same manner as cervical smears, dried and submitted to the laboratory.

Breast: Nipple discharge: A few drops should be expressed by pressing the subareolar region. The drops are touched on to a clean glass slide and spread with the help of another slide. Smears should be wet fixed in alcoholic fixative and submitted.

FINE NEEDLE ASPIRATION BIOPSY

Introduction: Fine needle aspiration biopsy is an inexpensive and rapid method of establishing the diagnosis of lesions and masses in various organs. It has advantages over other forms of biopsy because it is safe, rapid, reliable and relatively painless. This technique can be used to sample superficial and subcutaneous lesions in breast, thyroid, lymph node, salivary gland and superficial abdominal masses. It can also be used in deep visceral lesions with the help of radiologists using fluoroscopy, computer assisted tomography, angiography and ultrasonography to localise the lesions.

Technique: Proper clinical history is helpful in establishing diagnosis. To avoid any mishap during procedure, the patient should be explained and assured about the procedure. A disposable syringe with 21-25 gauge needle can be used for this purpose (5-10 ml). The area is cleaned thoroughly with a spirit swab. The needle is introduced into the mass negative pressure is applied by retracting the plunger and mass is probed in several directions (Figure 55.1). Prior to withdrawal of needle, the plunger is released allowing equalisation of pressure. A cotton swab is applied for few min to stop the bleeding.

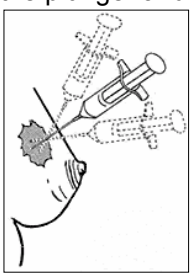


Figure 55.1: Fine needle biopsy technique

Slide Preparation: After aspiration the syringe is detached from the needle and filled with air. The syringe is reattached to the same needle and plunger is pushed to gently express the material onto glass slides. This step is repeated once or twice. The material is gently spread on the slides by using another slide. Some people use opposite surfaces of the two slides (one slide containing aspirated material and another clean slide) for smearing. The slides are allowed to air dry or fixed wet in solution containing ether and alcohol in equal proportions or 95% alcohol. The slides can be stained by Papanicolaou (PAP), Haematoxylin and Eosin (H&E),

Leishman and modified Giemsa stains (Figure 55.2).

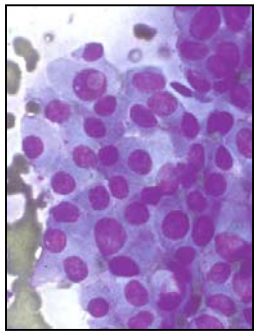


Figure 55.2: Fine needle aspiration smear stained with Giemsa

Pitfalls: If the specimen is too bloody, the cells from the actual lesion will be diluted in the smear. If the aspiration time is too long the specimen will clot in the syringe and needle and therefore will not be expelled or spread properly. If slides are incorrectly made, the nuclear details will be poor and artefacts created.

Complications: The major complications of aspiration biopsy of superficial lesions are bleeding and infection. In cases of deep-seated lesions pneumothorax, embolism, neurovascular injury, internal major bleeding or intraperitoneal leak of hollow viscus has been reported. Vasovagal syncope may also occur. Tumour spread along the needle tract has been reported following biopsy with large bore needles. However there is no evidence that aspiration using fine needles increases the risk of tumour dissemination. Those individuals who do the procedure often and are experienced in the method should perform this procedure. They may be pathologists, surgeons or radiologists.

56. HISTOTECHNOLOGY

Histological technique deals with the preparation of tissues for microscopic examination. The aim of good histological technique is to preserve microscopic anatomy of the tissues, and make them hard, so that very thin sections (5 micron) can be made. After staining these sections should represent the anatomy of the tissue as closely as possible to their structure in life. This is achieved by passing the total or selected part of the tissue through a series of processes. These processes are:

- Fixation
- Dehydration
- Clearing
- Embedding
- Cutting
- Staining

FIXATION

This is the process by which the constituents of the cells and tissues are fixed in a physical and partly also in a chemical state, so that they will withstand subsequent treatment with various reagents with a minimum loss of architecture. This is achieved by exposing the tissue to chemical compounds called fixatives.

MECHANISM OF ACTION

Most fixatives act by denaturing or precipitating proteins, which then form a sponge or meshwork, tending to hold the other cell constituents. Good fixation is the most important factor for the production of satisfactory results in histopathology. Following factors are important.

- Fresh tissue
- Proper penetration of tissue by fixative
- Correct choice of fixative

The inadequate penetration of fixative is one of the commonest causes of bad results. It is a rule that no fixative will penetrate a piece of tissue thicker than 1 cm. For dealing with specimens thicker than this, following methods are recommended.

Solid organs: Cut slices as big as necessary but not thicker than 5 mm.

Brain: For fixing the uncut brain, pass a thick thread under the vessels at the base of the brain. The organ is gently lowered into a bucket containing the solution and allowed to float with the help of thread.

Hollow organs: Either open and fill with fixative or pack lightly with wool soaked in fixative.

Small biopsies: In order to preserve the tissue in original orientation, it is better to place it first on a piece of filter paper and then put in the solution.

Large specimens, which require dissection: Inject fixative along the vessels or bronchi as in case of lung so that it reaches all parts of the organ. For lungs it is best to fill the bronchi with fixative. Putting the container at a place higher than the organ can do this. The fluid inflates the bronchi under gravity. After the lung has been inflated, it is put in a large bucket containing the fixative solution.

PROPERTIES OF AN IDEAL FIXATIVE

1. Prevents autolysis and bacterial decomposition.
2. Preserves tissues in their natural state and fixes all components (protein, carbohydrates, fats).
3. Makes the cellular components insoluble to reagents used in tissue processing.
4. Preserves tissue volume.
5. Avoids excessive hardness of fixed tissue.
6. Allows enhanced staining of tissues.
7. Is be non-toxic and non-allergic for user.
8. Is not be very expensive.

AMOUNT OF FIXING FLUID

This should be approximately 10-20 times the volume of the specimen.

CLASSIFICATION OF FIXATIVES

1. **Tissue fixatives**:
   a. Buffered formal saline
   b. Buffered glutaraldehyde
   c. Zenker's formal saline
   d. Bouin's fluid
2. **Cytological fixatives**:
   a. Ethanol
   b. Methanol
   c. Ether
3. **Histochemical fixatives**:
   a. Formal saline
   b. Cold acetone
   c. Absolute alcohol

COMMON FIXATIVES

Routine formalin: Formalin is sold as 40% W/W

solution of formaldehyde gas in water. It is used as 10% or better 15% solution (V/V) in normal saline, or calcium chloride solution. It does not precipitate protein but combines with NH_2 group to form an insoluble gel. It preserves practically all elements including fats and keeps phospholipid insoluble in fat solvents. It is the cheapest and most popular fixative.

Buffered formalin: Routine (10%) formal saline has an acidic pH, which results in formation of haematin crystals in the tissues. These crystals also interfere with staining. It is recommended that any fixative used must have a neutral pH. For this purpose phosphate buffers are added to the fixative. To prepare 10% buffered formal saline mix the following:

Pure formalin	10 ml
Sodium dihydrogen phosphate	0.4 g
Disodium hydrogen phosphate	0.65 g
Normal saline up to	100 ml

Advantages of buffered formalin: Buffered formalin has the following advantages:

1. Tissues can be left in fixative for long period of time e.g., one year.
2. There is no damage or hardening of tissue.
3. Sectioning is easy.
4. No haematin crystals are formed.
5. A number of staining procedures can be used.

Ethyl alcohol: It is used in 90-100% strength. It precipitates albumin and globulin but not nucleoproteins. It causes shrinkage and hardening of tissues. It destroys mitochondria. It is a reducing agent and, therefore, cannot be used with chromic acid, chromates and osmium tetraoxide. It preserves glycogen and is useful for histochemistry (glycogen, uric acid and iron) etc.

Mercuric chloride: It is used as a saturated (70%) or half saturated aqueous solution. It penetrates rapidly, precipitates proteins, fixes chromatin well and enhances its subsequent staining capability. It is rarely used alone but it is valuable for nuclear fixation.

Picric acid: It is used as a saturated aqueous solution (1%). Its penetration is poor and causes shrinkage but does not harden. It preserves glycogen and nearly all other elements. It does not affect the staining. It is not used alone.

Chromic acid: It is used either as a pure chemical or as a mixture of dichrome and acetic acid (e.g., in Zenker's solution). It is an oxidising agent and therefore incompatible with formalin or alcohol. It preserves most elements. It tends to weaken nuclear staining by dissolving nucleoproteins.

Potassium dichromate: It is used as 2-3% aqueous solution. It is a weak oxidising agent and tends to dissolve chromatin. It is a good cytoplasmic but bad nuclear fixative. It gives chromaffin reaction.

Osmium tetraoxide (Osmic acid): It is used as 2% aqueous solution. It is expensive and unstable. It is rapidly converted to vapours, which are irritating. It is a powerful oxidising agent. It penetrates very badly. It preserves fat and gives a black precipitate of osmium dioxide with unsaturated fats. Also preserves very fine cell details e.g., Golgi apparatus etc.

B-5 Fixative: It is used for fixation of lymph nodes. Its preparation is as follows:

Solution-A:

Mercuric chloride	6 g
Anhydrous sodium acetate	1.25 g
Hot distilled water	90 ml

Store at 4°C.

Solution-B:

10% Buffered formalin

Add 1 ml of solution B to 9 ml of solution A prior to use. Fix thin blocks for 2-4 hours, rinse and transfer to 70% ethyl alcohol for storage prior to dehydration and impregnation. Mercury crystals must be removed before staining with the help of iodine solution followed by sodium thiosulphate solution.

Zenker's Solution: It is used for bone marrow trephine biopsy and Negri bodies.

Stock Solution:

Potassium dichromate	25 g
Mercuric chloride	50 g
Distilled water up to	1L

It takes 24 hours to dissolve completely.

Working Solution: Working solution is made just before use by adding 5 ml of glacial acetic acid to 95 ml of stock solution.

FACTORS AFFECTING FIXATION

1. Size and thickness of the piece of tissue
2. Tissues covered by large amounts of mucus or blood, or organs containing very large amount of blood fix slowly.
3. Fatty and lipomatous tissues fix slowly.
4. Fixation is accelerated by agitation.
5. Fixation is accelerated by maintaining temperature around 60°C.

FROZEN TISSUE

Rapidly freezing the tissue is an alternative to fixation. This prevents autolysis and putrefaction. This is done on fresh tissue. Thin slices of tissue are placed in isopentane (OCT), which is cooled to -150°C by immersing in liquid nitrogen. This causes rapid freezing of tissue and prevents the formation of ice crystals within the cell. The frozen tissue is stored at -70°C. The frozen tissue retains all its antigens on the

cell membrane.

TISSUE PROCESSING

In order to cut thin sections of the tissue, the tissues must have a suitable hardness and consistency when presented to the knife-edge. These properties can be imparted by infiltrating and surrounding the tissues with paraffin wax, celloidin or low viscosity nitrocellulose (LVN), various types of resins or by freezing. The process is called tissue processing. It is done in stages. It can be sub-divided into dehydration, clearing, impregnation and embedding. It is important that all specimens are properly labelled before processing is started. For labelling, pen containing ordinary ink should not be used. Printed, graphite pencil written, type written or India ink written labels are satisfactory. Tissues that are fixed in osmium tetraoxide should be labelled on jar, as osmium tetraoxide will turn the label black. The label should be clearly written and must contain, in block letters, all necessary information. A system of transportation is required to carry the tissue through various steps in processing. The representative sections or entire biopsy specimen, when of small size are put in muslin cloth together with their label and are then transported from reagent to reagent in metal containers that have perforated walls, so that the reagent enters into the tissues. Alternatively labelled plastic cassettes with perforated walls are used to carry the sections. Tissue processing is a long procedure and requires 24 hours. Alternatively labelled plastic cassettes with perforated walls are used to carry each section. Processing of tissue can be done:

1. **Manually**: In which the tissue is moved from one container of reagent to another by hand. Agitation is also done manually.
2. **Automatically**: In which the same steps are completed automatically by a mechanical device. Now automatic tissue processors are available (Figure 56.1). In these processors there are different jars containing reagents.

Figure 56.1: Automatic tissue processor

These are arranged in a sequence. A mechanical device moves the tissue from one jar to another. Agitation is also done mechanically. Timings are controlled by a timer, which can be adjusted in respect of hours and min. Temperature is maintained around 60°C in jars containing paraffin wax. The steps involved in processing, whether done manually or mechanically, remain the same and are as under:

Dehydration: Using increasing strengths of alcohol e.g., 70%, 90% and absolute alcohol, dehydrates tissues. The duration for which tissues are kept in each strength of alcohol depends upon the size of tissue, fixative used and type of tissue. After fixation in aqueous fixatives delicate tissues need to be dehydrated slowly starting in 50% ethyl alcohol directly whereas most tissue specimens may be put into 70% alcohol. Delicate tissues will shrink too much when exposed to a high concentration of alcohol.

For routine, sections no thicker than 7 µm, the following scheme may be followed:

1. 70% alcohol - Methylated spirit for 1 hour
2. 90% alcohol - Rectified spirit 2 changes for 2 hours each
3. 100% alcohol - Absolute alcohol 2 changes for 2 hours each

In the above process dehydration is helped by agitation of the tissues hence duration is 2 hours. If not agitated, it may take much longer for the procedure. In the absolute alcohol chamber 1/2-1 inch thick layer of anhydrous copper sulphate separated by filter paper may be used. It takes away the water derived from the tissues. The volume of alcohol should be 50-100 times that of tissues. If this is not possible then frequent changes may be used.

Clearing (To remove alcohol): During dehydration the water in the tissue has been replaced by alcohol. In the next step alcohol is to be replaced by wax. As wax is not alcohol soluble, we replace alcohol with a substance in which wax is soluble. This step is called clearing. Clearing of tissues is achieved by immersing the tissue in any of the following substances.

1. Xylene
2. Chloroform
3. Benzene
4. Carbon tetrachloride
5. Toluene

Xylene is commonly used. Small pieces of tissue are cleared in 1/2-1 hour, whereas large (5 mm or more thick) are cleared in 2-4 hours. Cedar wood oil can also be used. It is an excellent clearing agent and tissues may be kept for months in it without hardening. However it is slow in action and extra time is required in molten wax.

Impregnation with Wax: This is allowed to occur at melting temperature of wax, which is 54-60°C. Volume of wax should be about 25-30 times the volume of tissues. For better results, impregnation is done serially in 3-4 jars, however 2 jars are sufficient. The duration of impregnation depends on the size and type of tissue and the clearing agent employed. Longer periods are required for larger pieces and also for harder tissues like bones and skin as compared to liver, kidney, spleen, lung etc. Xylene is easiest to remove and 1-2 changes of wax are sufficient. Total duration of 4 hours is sufficient in all the jars for routine processing. Types of waxes employed for impregnation are:

1. **Paraffin Wax**: It is used routinely. It has hard consistency, so sections of 3-4 micron thickness can be cut.
2. **Water-soluble Wax**: It has the advantage that the tissue can be directly placed in it, without dehydration and clearing. However the disadvantage is that fragmentation of the section takes place in the floating bath.

Other materials used for impregnation are:

1. **Celloidin**: The consistency of celloidin is rubbery so it can be used for hard tissues like bone. High temperature is not required during processing so tissue shrinkage does not take place.
2. **Gelatin**: This is used for embedding friable tissue. It has the advantage that creases can be removed easily.
3. **Paraplast**: This material is the combination of paraffin wax and several plastic polymers. Its consistency is softer than paraffin and its sections are free from any wrinkles. Its melting point is 56°C. Another substance called paraplast plus is superior because its penetration is more, and this reduces the processing time.

Casting or Blocking: Embedded tissues are placed in a mould, which may be metal or plastic with their label and then fresh molten wax is poured in it and allowed to settle and solidify. Care is taken not to allow any bubbles to form. Once the block has cooled sufficiently to form a surface skin it should be immersed in cold water to cool it rapidly. Failure to do this will often cause crystallisation of wax. After the block has completely cooled it is cut into individual blocks and each is trimmed. The labels are made to adhere to the surface of the block by melting the wax with a metal strip sufficiently warmed.

Summary of Paraffin Wax embedding:

Dehydration	Long time routine	Short time routine
70% Ethanol	1 hour	15 min
90% Ethanol I	2 hour	20 min
90% Ethanol II	2 hours	20 min
100% Ethanol I	2 hour	20 min
100% Ethanol II	2 hours	20 min
Clearing		
Xylene I	1 hours	20 min
Xylene II	1 hours	20 min
Wax impregnation		
Paraffin wax I	2 hour	40 min
Paraffin wax II	2 hour	40 min

STAINING

Staining is a process by which a colour is imparted to sectioned tissue. Specially manufactured dyes are used for this purpose. These dyes are prepared by adding an auxochrome to a chromophore. An auxochrome is a compound which when added to a chromophore forms a dye. This may be acidic or basic. A chromophore is a compound, which although coloured, does not have the properties of a dye or stain. The dye stains the tissues by binding with specific sites. Compounds called mordants help in achieving this binding.

CLASSIFICATION OF STAINS:

All stains are composed of an acid and a basic component. Generally the stains are classified as:

- Acid stains
- Basic stains
- Neutral stains

Acid stains: In an acid stain the acidic component is coloured and the basic component is colourless e.g., in acid fuchsin, which is composed of sodium and rosaniline trisulphonic acid, the sodium is colourless and rosaniline trisulphonic acid is coloured. Acid dyes stain basic components of tissue e.g., cytoplasmic proteins. The colours imparted are shades of red. Most commonly used acid dye is eosin.

Basic stains: In the basic dyes the basic component is coloured and the acidic component is colourless. The example is basic fuchsin. Basic dyes stain acidic components of tissue e.g., nucleic acids. The colours imparted are shades of blue. Most commonly used basic dye is haematoxylin.

Neutral stains: When an acidic dye is combined with a basic dye a neutral dye is formed. As it contains both colouring components it stains all components of tissue but with different colours. This is the basis of Romanowsky stains (e.g., Leishman stain).

PROCEDURE OF STAINING

Like processing, staining can also be performed manually or mechanically.

Manual staining: In a small laboratory where only a few slides are stained this is the method of choice. It is time consuming, but economical. Reagent containers are placed in a sequence. Slides are placed in a carrier and are then moved from one container to other at specified intervals till the process is complete.

Automated staining: The above procedure is performed with the help of a mechanical device similar to one described for processing. Automated stainers of various kinds are now freely available. In these the reagent jars are arranged according to a desired sequence. The carrier containing slides is rotated through these at intervals, which are set by the operator (Figure 56.2). These are usually microprocessor controlled and are programmable.

Figure 56.2: Automatic stainer

The advantages are:

- Reduce manpower requirements
- Precise control the timing
- Large number of slides stained simultaneously
- Less reagent consumed

HAEMATOXYLIN AND EOSIN STAINING

It is commonly used for routine histopathology and in diagnostic cytology. Its particular value lies in its ability of imparting proper differentiation to distinguish between different types of connective tissue fibres and matrices, by staining them different shades of red and pink.

Principle: First the tissue is cleared of all wax and then rehydrated to facilitate the entry of dyes. The tissue sections are then sequentially exposed to a basic dye e.g., Harris's Haematoxylin and an acid dye e.g., eosin. This stains both basic and acid components of the tissue.

Reagents:

Harris's Haematoxylin:

Haematoxylin crystals	5.0 g
Alcohol 95%	50 ml
Ammonium or Potassium Alum	100 g
Mercuric oxide	2.5 g
Distilled water	1 litre
Glacial acetic acid	40 ml

Dissolve separately by heating, haematoxylin in alcohol and alum in water, mix and rapidly boil. Remove from flame and add mercuric oxide. Reheat for 1 min or until it becomes dark purple. Remove from flame and cool in a basin of cold water. Stain is ready to use. Add 2-4 ml of Glacial acetic acid per 100 ml of solution if desired.

Acid alcohol: Mix one litre 70% alcohol with 10 ml of concentrated hydrochloric acid.

Ammonia water: Mix 2-3 ml of strong ammonia with one litre of tap water.

Alcoholic eosin solution:

Eosin (water soluble)	2 g
Distilled water	160 ml
Alcohol 95%	640 ml

Other reagents: Xylol, absolute alcohol, rectified spirit and methylated spirit are also needed.

Staining procedure

1. Put the sections fixed on a glass slide in xylol for 3 min.
2. Then transfer to absolute alcohol for 3 min.
3. Transfer to rectified spirit (80% alcohol) for 2 min.
4. Place in methylated spirit for 2 min.
5. Wash the slide in running water for 1 min and put it in Harris haematoxylin for 3-5 min.
6. Wash in running water for 30 seconds and wash the excess dye in 1% acid alcohol by continuous agitation for 15 seconds.
7. Wash in running water for 30 seconds.
8. Give 2-3 dips in ammonia water solution until tissues attain a blue colour.
9. Wash in running water for 2-3 dips.
10. Counter stain with eosin for 2-3 min.
11. Wash in running tap water for 30 seconds.
12. Dehydrate by keeping in increasing concentrations of alcohol (2-3 dips in 70%, 95% and absolute alcohol).
13. Clear it in xylol and mount with Canada balsam.

Result

Nuclei	Bright blue
Muscle, keratin	Bright pink
Collagen and cytoplasm	Pale pink
Erythrocytes	Orange red

Notes and Precautions

Other haematoxylins like Mayer's haematoxylin may also be used. All have different methods of preparation. The reagents must be checked daily for deterioration and changed when needed. In the manual method, the xylol and alcohols must be changed daily, haematoxylin once a week, eosin and acid alcohol twice a week, and ammonia water daily. This regimen may be modified by the amount of usage. In the automatic stainer, xylol, alcohols, eosin and acid alcohol, are changed twice a week. Haematoxylin is changed once in two weeks and ammonia water is changed daily. The quality of alcohol

must be checked before use. This can be done by adding 4-5g of copper sulphate crystals to a Coplin jar containing alcohol. If the colour remains unchanged (bluish white) for 10 min, it is acceptable. If the colour changes to green the quality of alcohol is unsuitable for processing.

57. SPECIAL STAINING TECHNIQUES

Special stains are used to identify certain normal and abnormal substances present in the cells and tissue, which cannot be differentiated by routine haematoxylin and eosin staining. For example Van Gieson's special stain is used to differentiate between connective tissue and muscle fibres. Similarly Pearl's stain is needed to demonstrate iron in the tissue.

INTERPRETATION AND QUALITY CONTROL

One must be experienced enough to interpret the results of special stains. Some times artefacts or faulty technique can give false results. It should be a policy that with every special staining procedure, both negative and positive known controls should also be stained. This will greatly help in interpreting the results of special stains. Following should be strictly observed:

1. Blocks and unstained control slides should be available for running controls.
2. All the reagents should be freshly prepared to get optimum results.
3. All the reagents should be stored in brown coloured bottles with tight stoppers.
4. All the reagent bottles should be properly labelled with expiry date clearly mentioned.
5. All the steps in special staining procedures should be meticulously followed. Special instructions about fixatives should be followed and any precautions mentioned should be taken.

PERIODIC ACID SCHIFF REACTION (PAS)

Purpose: It is required to demonstrate carbohydrates e.g., neutral muco-polysaccharides, mucoproteins and glycoproteins in the tissue. It can also be used to demonstrate fungi.

Principle: Aldehyde is generated by oxidation of 1:2 glycol groups present in carbohydrates by periodic acid. This combines with Schiff's reagent to form a coloured compound *in situ*.

Requirements

1. 0.1% periodic acid
2. 0.5% sodium metabisulphite
3. Schiff's solution (commercially available)
4. Haematoxylin as in H&E stain

Procedure

1. Rehydrate as for H&E staining.
2. Rinse in tap water for 5 min.
3. Rinse in distilled water by giving 15 dips.
4. Oxidise in 0.1% periodic acid for 15 min.
5. Wash well in tap water for 5 min.
6. Rinse well in 3 changes of distilled water giving 5 dips in each.
7. Treat with Schiff's reagent for 10 min.
8. Treat with 3 changes of 0.5% sodium metabisulphite for 2 min each.
9. Wash in tap water.
10. Stain in hematoxylin as desired.
11. Dehydrate, clear and mount as for H&E stain.

Result: Neutral mucopolysaccharides, mucoproteins and glycoproteins stain pink to magenta whereas nuclei stain blue. Fungi also stain magenta.

PERIODIC ACID SCHIFF WITH DIASTASE

Purpose: To stain glycogen

Principle: Glycogen is removed from the tissue by treating with diastase. As a result the tissue will not stain with PAS stain.

Requirements

1. Buffered saline prepared by dissolving 1g NaCl, 1.3 g disodium hydrogen phosphate and 0.8 g. dihydrogen sodium phosphate in 100 ml distilled water. Keep refrigerated.
2. 0.5% MALT diastase
3. Reagents for PAS stain

Procedure

1. Rehydrate the sections.
2. Rinse in warm water (37°C) by giving 10 dips.
3. Rinse in two changes of warm buffered saline by giving 10 dips in each.
4. Place in buffered saline for 1 hour at 37°C.
5. Place in 0.5% diastase for 1 hour at 37°C.
6. Rinse in absolute ethyl alcohol by giving 10 dips and proceed from step 4 onwards as for PAS stain.

Results: The tissue, which has given positive results with PAS stain, will become negative after treatment with diastase.

ALCIAN BLUE AND ALCIAN BLUE PAS COMBINED STAIN

Purpose: It is used for distinguishing between mucin-secreting adenocarcinoma and undifferentiated squamous cell carcinoma, and

to identify naturally occurring carbohydrates in tissue.

Principle: Connective tissue ground substance is coloured only with Alcian blue because it lacks sufficient polysaccharides to react with PAS. Epithelial mucin or glycogen will react with PAS and not at all with Alcian blue. Complex carbohydrates, such as epithelial mucin secretions, stain with both Alcian blue and PAS.

Control: Small intestine

Requirements

1. Alcian blue prepared by dissolving 01 g Alcian blue in 100 ml of 3% glacial acetic acid (pH should be 2.5).
2. 3% Glacial acetic acid prepared by diluting 3 ml glacial acetic acid in 97 ml of distilled water.
3. Nuclear Fast red counter stain prepared by dissolving 0.1g nuclear fast red, 5 g aluminium sulphate and one crystal of thymol in 100 ml distilled water. Heat water to 60°C and then add to it aluminium sulphate and stir until dissolved. Then add nuclear fast red and cool to 50°C. Filter and add thymol crystal. Store in refrigerator.
4. 1% Periodic acid
5. Schiff's reagent

Procedure for Alcian Blue stain

1. Rehydrate the section.
2. Rinse in tap water for 2 min.
3. Stain in Alcian blue for 20 min.
4. Rinse in tap water for 10 min.
5. Place in nuclear fast red counter stain for 3-5 min.
6. Rinse in tap water for 15 dips.
7. Dehydrate, clear and mount.

Results: Acid mucopolysaccharides stain blue whereas other tissue elements stain red.

Procedure for Alcian Blue-PAS combined stain:

1. Rehydrate the sections and rinse in distilled water
2. Treat with Alcian blue solution for 30 min.
3. Wash well with running tap water for 2 min.
4. Rinse in distilled water.
5. Treat with 0.5% Periodic acid for 10 min.
6. Wash in running tap water for 5 min.
7. Rinse in distilled water.
8. Treat with Schiff's reagent for 10 min.
9. Wash in running tap water for 10 min.
10. Stain with Mayer's Haematoxylin, differentiate and blue.
11. Dehydrate, clear and mount.

Results: Acid mucin is stained blue, neutral mucin is stained red and mixture of the two is stained purple.

GOMORI'S RETICULIN STAIN

Purpose: To stain reticulin fibres.

Principle: Silver oxide is precipitated on the reticulin fibres in the presence of ammoniacal solution.

Requirements:

1. Silver nitrate reagent: To 20 ml of 10% silver nitrate solution, add 4 -5 ml of 10% potassium hydroxide. With continuous shaking, add 28% ammonia water drop by drop, till the precipitate is dissolved. Now carefully add 10% silver nitrate solution drop by drop, till precipitate, which forms and easily disappears on shaking. Add an equal volume of distilled water. If stored in dark, can be used for 1-2 months.
2. Potassium permanganate 1%
3. Potassium metabisulphite 3%
4. Iron alum 2%
5. Formalin 10%
6. Gold chloride (0.2 g dissolved in 100 ml distilled water)
7. Sodium thiosulphate 3%

Procedure

1. Rehydrate the sections as usual.
2. Oxidise with 1% potassium permanganate for 1-2 min and rinse in tap water.
3. Decolourise with 3% potassium metabisulphite for 1 min and rinse in tap water.
4. Sensitise in 2 percent iron alum for 1 min.
5. Wash in tap water for 2-3 min, and then rinse in 2-3 changes of distilled water.
6. Impregnate in silver solution for 3 min.
7. Rinse in distilled water for 20 sec.
8. Reduce in 10 percent formalin for 3 min.
9. Wash in running water for 2-3 min and rinse in distilled water.
10. Tone in gold chloride (yellow) for 10 min in a Coplin jar.
11. Rinse in distilled water.
12. Reduce toning in 3% potassium metabisulphate for 1 min.
13. Rinse in distilled water.
14. Fix in 3% sodium thiosulphate for 1 min.
15. Wash in water.
16. Dehydrate in absolute alcohol, clear in xylene and mount in Canada balsam.

Results:

- Reticulin fibres stain black
- Collagen fibres stain purple
- Nuclei and cytoplasm stain shades of grey

MASSON'S TRICHROME STAIN

Purpose: To differentiate connective tissue elements

Fixative: The tissues should be fixed in Bouin's or Zenker's fluid. Formalin fixed sections should be mordanted in Zenker's fluid overnight at room temperature or at 56°C for one hour.

Requirements

1. Weigert's iron haematoxylin
   a. Solution A
      i) Iron hematoxylin 1.0 g
      ii) 95% alcohol 100 ml
   b. Solution B
      i) 29% aqueous Ferric Chloride 4 ml
      ii) Distilled water 95 ml
      iii) Concentrated HCl 1 ml
   c. Working solution: Mix equal parts of solutions A and B
2. Biebrich scarlet acid fuchsin solution: Prepared by mixing 1 ml glacial acetic acid, 10 ml 1% aqueous acid fuchsin in 90 ml of 1% aqueous Biebrich scarlet.
3. Phosphomolybdic-Phosphotungstic acid solution. Prepared by dissolving 2.5 g each of phosphomolybdic acid and phosphotungstic acid in 100 ml distilled water.
4. Aniline blue solution prepared by dissolving 2.5 g aniline blue in 2 ml acetic acid and 100 ml distilled water.

Procedure:

1. Bring sections to water as usual.
2. Rinse in distilled water.
3. Stain in Weigert's iron haematoxylin for 10 min.
4. Wash in running tap water for 10 min.
5. Rinse in distilled water.
6. Stain in Biebrich scarlet-acid fuchsin solution for 5 min.
7. Rinse in distilled water.
8. Place in aqueous phosphomolybdic acid-phosphotungstic acid solution for 10 min.
9. Drain slide, and pour on it aniline blue solution for 5 min.
10. Rinse in distilled water.
11. Differentiate in 1% acetic acid for 3 min.
12. Dehydrate in absolute alcohol clear in xylene and mount in Canada balsam or DPX.

Results: Nuclei stain blue black whereas cytoplasm, muscle and keratin granules stain red. Collagen, cartilage, mucin and basophil granules are stained blue.

VAN GIESON'S STAIN

Purpose: To differentiate between muscle and collagen fibres

Principle: Collagen fibres are stained red by picrofuchsin solution (Van Gieson's solution).

Requirements

1. Van Gieson's Solution: This must be prepared immediately before use. It is prepared by mixing 1 ml of 1% aqueous acid fuchsin (described earlier) and 4 ml of saturated aqueous solution of picric acid.
2. Weigert's Iron haematoxylin:
   a. Solution A
      i) Haematoxylin 1 g
      ii) Absolute alcohol 100 ml
   b. Solution B
      i) 29% aqueous ferric chloride 04 ml
      ii) Distilled water 95 ml
      iii) HCl concentrated 1 ml
   c. Working solution: Mix equal parts of solution A and B before use.

Procedure:

1. Use any fixative and prepare paraffin sections.
2. Bring sections down to water by passing slide through xylene, absolute alcohol, 95% alcohol and distilled water.
3. Stain nuclei with Weigert's haematoxylin solution for 10 min.
4. Rinse and decolourise with 1% acid alcohol, 1-2 dips.
5. Blue in ammonia water, 1-2 dips.
6. Wash in water, 1-2 dips.
7. Counter stain for 1-3 min in Van Gieson's solution.
8. Blot to dry.
9. Clear in Xylene, 1-2 changes.
10. Mount in Canada balsam.

Results: Collagen stains bright red, muscles and cornified epithelium stain yellow and nuclei stain blue black.

VERHOEFF'S ELASTIC STAIN

Purpose: To stain elastic fibres.

Principle: Elastic fibres are stained by Verhoeff's solution in the presence of ferric salts (Oxidisers).

Requirements

1. Verhoeff's Solution: The solution should be prepared fresh each time. To prepare Verhoeff's solution the ingredient must be added in the following order:
   a. 10 ml 5% alcoholic haematoxylin
   b. 4 ml 10% aqueous ferric chloride
   c. 4 ml Lugol's iodine solution (potassium iodide 4 g, Iodine 2 g in 100 ml water)
2. Ferric chloride 2%
3. Aqueous sodium thiosulphate 5%
4. Van Gieson's counter stain (saturated aqueous solution of picric acid 100 ml, 1% acid fuchsin 5 ml).

Procedure

1. Bring section down to water by passing

through Xylene, absolute alcohol, 95% alcohol and distilled water.
2. Rinse in running tap water for 3 min.
3. Stain in Verhoeff's solution till black (15 min).
4. Rinse in distilled water.
5. Differentiate in 2% ferric chloride for only a few dips until grey.
6. Wash in water.
7. Rinse in distilled water.
8. Place in 5% sodium thiosulphate for 1 min. Wash in tap water – 5 min.
9. Counterstain with Van Gieson's solution for 1/2-1 min.
10. Differentiate in 95% alcohol.
11. Dehydrate, clear and mount.

Results: Elastic tissue stains black, nuclei stain grey black, collagen stains red whereas other structures stain yellow.

BENNHOLD'S CONGO RED STAIN

Purpose: To demonstrate amyloid.
Principle: Amyloid is stained salmon red with Congo red solution in the presence of differentiating agent.
Requirements
1. Congo red solution (1 g Congo red in 100 ml distilled water)
2. Differentiating agent (1.3 g lithium carbonate in 100 ml distilled water)
3. Harris haematoxylin

Procedure
1. Bring section to water.
2. Rinse in distilled water for 5 min.
3. Stain in Congo red solution for 10-30 min.
4. Dip in saturated lithium sulphate solution for 15 sec.
5. Wash in running tap water for 15 min.
6. Counterstain with Harris haematoxylin for 1-2 min.
7. Differentiate in 1% acid alcohol.
8. Wash in water.
9. Blue in ammonia water
10. Wash in water.
11. Dehydrate, clear and mount.

Results: Under Bright Field Microscope amyloid appears salmon red and nuclei appear blue Under Polarised Light Microscope amyloid appears apple green, collagen yellow and nuclei blue.

VON KOSSA'S CALCIUM STAIN, MODIFIED

Purpose: To demonstrate $Ca_3(PO_4)_2$ and $Ca(CO_3)_2$ in tissue.
Requirements
1. 5% aqueous silver nitrate
2. 5% aqueous sodium thiosulphate
3. Nuclear fast red stain. Prepared by dissolving 0.1 g nuclear fast red powder in 100 ml of 5% aqueous aluminium sulphate solution with aid of heat. The solution is then cooled, filtered and a crystal of thymol is added.

Procedure
1 Bring section to water.
2 Rinse in three changes of distilled water for 10 dips each.
3 Place in 5% silver nitrate for 30-60 min. in direct sun light.
4 Rinse in 5 changes of distilled water, 10 dips each.
5 Place in 5% aqueous sodium thiosulphate for 2-3 min.
6 Wash in distilled water.
7 Counter stain in nuclear fast red for 5 min.
8 Wash in distilled water.
9 Dehydrate, clear and mount.

Results: Calcium appears black and nuclei appear blue, whereas cytoplasm is stained pink.

PAPANICOLAOU STAINING

Purpose: To stain the cells in cervico-vaginal and sputum smears for cytology, also used for staining of fine needle aspiration smears.
Principle: Nuclei are stained blue by haematoxylin; cytoplasm is stained green by EA 50 or by orange G depending upon maturity of cells.
Requirements
1 Harris's haematoxylin
2 Orange G (OG-6)
3 EA 50
4 70% alcohol
5 95% alcohol
6 0.1% Ammonia

Procedure
1 Remove slide from fixing jar and pass through descending grades of alcohol to water.
2 Stain in Harris haematoxylin for 5-10 min.
3 Rinse in tap water.
4 Differentiate in 1% acid alcohol.
5 Blue in ammonia water.
6 Wash in tap water.
7 Dip in 70% alcohol for 2 min.
8 Place in 95% alcohol for 2 min.
9 Stain in orange G for 5-7 min.
10 Rinse in two changes of 95% alcohol.
11 Stain in or EA 50, 5-7 min.
12 Rinse in two changes of 95% alcohol.
13 Rinse in two changes of absolute alcohol.
14 Drain and clear in xylene and mount.

Results: Nuclei stain blue. Cytoplasm stains varying shades of pink, blue, yellow or green in

increasing order of maturity. Trichomonas if present will stain pale greenish blue.

MODIFIED ZIEHL-NEELSEN STAIN

Purpose: To demonstrate Mycobacterium tuberculosis

Principle: M. tuberculosis will retain carbolfuchsin in the presence of decolouriser hydrochloric acid.

Requirements

1 Carbol fuchsin stain: See page 161.
2 Differentiating solution: 1% HCl (1 ml HCl in 99 ml 70% ethyl alcohol).
3 Methylene blue counter stain: 0.5 g methylene blue and 0.5 ml concentrated glacial acetic acid in 99.5 ml of distilled water.

Procedure

1 Bring section to water.
2 Wash in tap water for 5 min.
3 Stain in preheated carbol fuchsin (60°C) for 3 min.
4 Rinse well in tap water.
5 Place in differentiating solution (1% HCl) until the sections are pale pink.
6 Rinse in tap water for 5 min.
7 Place in methylene blue solution for 15-30 sec.
8 Rinse in distilled water.
9 Dehydrate, clear and mount.

Result: Acid-fast bacilli (AFB) stain red against a blue background of nuclei and other tissue elements.

MAY-GRUNWALD-GIEMSA STAIN

Purpose: To demonstrate *Giardia lamblia*, *Toxoplasma gondii* and haematopoietic tissue

Requirements

1 Jenner's solution. Mix equal volumes of Jenner's stock solution (1 g Jenner's stain in 400 ml methyl alcohol) and distilled water.
2 Giemsa stain. Mix 1g Giemsa powder in 66 ml glycerine. Place in the oven at 60°C for 2 hours. Add 66 ml methyl alcohol and mix well.
3 1% Rosin differentiating solution
4 Buffered distilled water, *p*H 7.0.

Procedure

1 Bring section to water.
2 Rinse in distilled water for 5 min.
3 Place in 3 changes of methyl alcohol each for 5 min.
4 Place slides in Jenner's solution for 6 min.
5 Rinse in buffered water solution and blot.
6 Place slides in Giemsa stain for 30-40 min.
7 Rinse quickly in Rosin differentiating solution 1-5 dips.
8 Rinse in distilled water.
9 Dehydrate quickly, clear and mount.

Results:

Nuclei blue
Cytoplasm pink to rose
Bacteria pale blue

PERL'S STAINING REACTION

Purpose: To demonstrate ferric iron in haemosiderin and asbestos bodies

Principle: Iron is stained by potassium ferrocyanide in the presence of HCl.

Requirements

1 Perl's solution. Prepared just before use by mixing equal parts of 2% HCl and 2% potassium ferrocyanide.
2 Counter stain. 1.5 ml 0.5% basic fuchsin and 3 ml 1% neutral red in 100 ml distilled water.

Procedure

1 Bring section to water.
2 Rinse in 2 changes of distilled water, 15 dips each.
3 Place in Perl's solution for 45 min.
4 Rinse in 2 changes of distilled water, 15 dips each.
5 Place in counter stain for 3 min.
6 Rinse in distilled water, 20 dips.
7 Dehydrate, clear and mount.

Result

Haemosiderin: blue
Nuclei/other tissue elements: red

FROZEN SECTION[1]

Frozen section is a technique in which tissue is frozen rapidly to temperature of -20°C and then sections are cut on a cryomicrotome and stained. In this way tissue can be examined microscopically within 5-10 min of its removal from the body. Frozen section has the advantage that it reduces the time of processing from 18 hours to 5 min. It has the disadvantage that only 8-10 µm thick sections can be cut and finer details of tissue cannot be examined. Frozen section is performed on a machine called cryostat or freezing microtome (Figure 57.1). Following are the situations where frozen sections are helpful.

Figure 57.1: Cryostat

[1] Frozen section is an emergency situation. Specimens should be dealt with immediately. Fresh reagents should be used for staining.

1. When a rapid diagnosis regarding benign or malignant nature of lesion is required to decide the extent of surgery while the patient is still on the operating table.
2. When study of fats, proteins or antigenic markers is required as routine processing of tissue destroys them.
3. When the type/nature of tissue is to be determined in a biopsy material.

Precautions

1 Laboratory workers should always be informed about frozen section before hand.
2 All preparations should be completed before arrival of the specimen.
3 Cryostat should preferably remain "ON" all the time to maintain its temperature at -20°C.
4 The tissue should be dealt with immediately on its arrival in the laboratory.

Procedure

1 A pathologist performs gross examination of the tissue. He then takes representative sections. If tiny fragments are received, the tissue is processed as such.
2 Tissue is then placed on a metallic block and is covered with appropriate amount of OCT compound (Isopentane). OCT compound has the property to freeze rapidly at -20°C.
3 The block holder is placed over the freezing stage of cryostat and the glass door of cryostat is closed to maintain its temperature.
4 OCT compound along with tissue is frozen within 1-2 min. The door of cryostat is opened. The block holder is transferred to its stage and fixed.
5 The block and knife is sprayed with cryo-freezer spray to maintain temperature.
6 The block is trimmed with cutting mechanism adjusted at 35 µm thickness.
7 Before cutting the actual sections, the **anti roll plate** is replaced. This is a glass plate applied over the external surface of knife to prevent rolling of cut sections. 8-10 µm thick sections are cut. In case of fatty tissue 15 µm thick sections are cut.
8 Sections are transferred to slides, which are then rapidly taken to the staining rack. Routinely, frozen sections are stained with **rapid haematoxylin eosin staining** as follows:
   a. Dip the slide in tap water once.
   b. Dip in Harris haematoxylin for 1-2 min.
   c. Rinse in tap water.
   d. Differentiate in 1% acid alcohol, one dip only.
   e. Blueing is done in ammonia. One or two dips only.
   f. Rinse in running tap water.
   g. Dip in eosin for 30 seconds to 1 min.
   h. Rinse in water.
   i. Dehydrate through 70%, 80%, 90% alcohol. One to two dips in each.
   j. Dip in absolute alcohol for 1 min.
   k. Dip in xylol for 1-2 min for clearing.
   l. Mount with Canada Balsam..

58. POSTMORTEM EXAMINATION

Postmortem examination (**autopsy**) is the examination of the dead body. A pathologist performs this examination. It includes **external** examination and **internal** examination i.e., dissecting the body to see the internal organs and cavities. Postmortem is carried out in a place called **Mortuary**.

OBJECTIVES

There are mainly two reasons for performing a postmortem.

- Medico-legal
- Medical

MORTUARY (POSTMORTEM ROOM)

Mortuary must provide optimum conditions in order to produce good results from post-mortem (Figure 58.1).

1 It should be properly sized, well lighted, well equipped and well covered.
2 It should have proper antiseptic conditions.
3 The recommended **equipment** for a mortuary is:
4 Autopsy table
5 Dissecting table
6 Head block
7 Sponge basin
8 Scales or spring balance to weigh up to 5 Kg.
9 Sink, wide and shallow with water tap.
10 Two buckets.

Figure 58.1: A standard autopsy room

Following **instruments** are required for postmortem:

- Scalpel with blades, three in number
- Knife with 25-27x2.5 cm blade and blunt end for sectioning of organs
- Knife with 12-13x1.5 cm blade and sharp end for removing larynx and pelvic viscera.
- Knife hernia (probe pointed bistoury) for opening heart and for blind dissection.
- Forceps bone holding of lances 38.75 cm (15.1 inch).
- Forceps, dissecting, toothed 17.5 cm
- Forceps dissecting plain 12.5 cm
- Forceps gouge simple embalming type
- Scissors small flat, fine pointed (Strabismus pattern)
- Scissors flat (Mayo's) 18.75 cm (PVMS[1] No.05646)
- Scissors bowel (one blade longer than the other)
- Chisel bone .78 cm (PVMS No.05156)
- Chisel bones 1.9 cm (PVMS No.5157)
- Mallet, carpenter's wooden
- Saw, amputating Butcher's with 25 cm blade
- Probe, silver malleable 25 cm
- Foley's catheter
- Measuring Jug or cylinder (1-2 litre capacity)
- Needle Sail maker's
- Electric saw and accessories
- Goggles
- Heavy gloves
- Miscellaneous Equipment: Apron, rubber gloves sizes 6-8, masks, soap, sponges (natural), specimen jars, sterilised test tubes, disposable syringes 20, 10 and 5 ml, 10% formal saline, sterile swabs and microscopic slides etc. A container with saturated solution of sodium chloride for placing tissue for chemical examination is also required.

DOCUMENTS REQUIRED

Following documents must be completed before postmortem.

1. Death certificate stating date and time of death and probable cause of death.
2. Identification certificate of dead body by a competent and reliable person whose full particulars are noted down.
3. Authorisation papers from the Commanding officer/medical superintendent of the hospital or other competent authority to perform postmortem.

[1] PVMS = Priced Vocabulary of Medical Stores

4. Consent for postmortem from the relatives, Commanding Officer or medical superintendent of the hospital.
5. If the deceased was admitted in a hospital before death full clinical information i.e., hospital papers.
6. In case of sudden death, a preliminary report of the examination of the site where the body was found and all relevant information.

ESSENTIALS DURING POSTMORTEM

1. No relative of deceased or investigating officer should be present inside the mortuary.
2. All belongings of the dead body e.g., clothing, ornaments, cash, diary etc. should be examined, noted, and handed over to hospital authorities after taking a receipt in writing.
3. Any bullet, pellets or any other remains of weapon of violence found during postmortem should be secured, packed, sealed and signed. A full note of these to be made in the report. The material should be handed over to the hospital authorities after taking a receipt.
4. The dead body should be handled gently and given all respect.
5. Information dictated by the pathologist should be noted with great care.
6. Specimens for microbiological examination should be collected by aseptic technique in sterile containers.

AUTOPSY TECHNIQUE

Extent of autopsy is determined by the clinical history. In addition to the determination of cause of death it should be able to demonstrate major pathological changes. The anatomical findings should be consistent with clinical data and any major discrepancy should be explained and noted. Only a competent pathologist should perform the autopsy. Technicians can only assist. The date and time of autopsy should be noted before proceeding. The method described below applies to a routine autopsy, which can be modified to suit the requirements of a particular case.

EXTERNAL EXAMINATION

Before starting postmortem, external examination of the dead body is carried out. Following points are noted.

1. Sex and probable age of the deceased.
2. Body length, built and state of nutrition of the deceased.
3. Rigor mortis: This is postmortem rigidity at the joints due to muscle contraction. Its presence or absence is noted.
4. Postmortem lividity: This is the bluish discoloration of skin on dependent parts e.g., back if the body is lying supine. Its presence or absence is noted.
5. The colour, morphology and distribution of any rash or pigmentation, old or recent signs of injury or surgery are recorded.
6. Appearance of cornea, whether hazy or not, and whether the pupils are dilated or normal or constricted.
7. Body orifices e.g., mouth, nostrils, anus, urethra are examined. Whether these contain any extraneous material or blood etc., or not.
8. Neck is examined for ligature or finger marks.
9. Any mark of violence of any kind anywhere on the body.
10. Any wound or bullet injuries or deformities are noted including their exact site, size and shape. In case of bullet injuries wound of entry and wound of exit are differentiated. Wound of entry is usually small with clean margins whereas wound of exit is large with torn margins. The area around the wound is examined for burns, gunpowder etc. The track of the wound is examined by passing a probe through it.
11. Any fracture deformity or scar mark is noted.

INTERNAL EXAMINATION

Primary Incision: A primary incision is given on the body to cut through skin, subcutaneous tissue and muscle. This exposes the thoracic and abdominal cavity. Two types of incisions are usually given.

1. **T-shaped incision**: A central incision starting from jugular notch and extending down to the symphysis pubis passing along one side of the umbilicus. Second incision starting from the tip of one shoulder, extending along the clavicles up to the tip of the other shoulder. This makes it a T-shaped incision. This incision is preferred for cosmetic reasons.
2. **Y-shaped incision**: In this type the central incision is the same, but from the jugular notch it extends towards middle of each clavicle, then along the sternomastoid reaching behind each ear. The primary incision should be deep enough to cut through skin, subcutaneous tissue, fat and muscle. But it should not injure the intercostal spaces, ribs or cartilage,

peritoneal membrane and viscera in thoracic or abdominal cavity. This incision is preferred for ease for removal of organs, especially from the neck.

3. **Opening of Thorax**: Once the primary incision has been made the flap of skin, subcutaneous fat and muscle is dissected as close to the ribs as possible. The cutting edge of the knife should be almost perpendicular to the course of the ribs. The dissection, as a rule, should start from the costal margin and is continued until the tendons of sternocleidomastoid muscles are visible. The flap is then raised and thoracic cage is exposed. If pneumothorax is suspected some water is put between the skin flap and thoracic wall. The chest wall is punctured under water with the help of a scalpel. Pneumothorax will be revealed by appearance of air bubbles. With the help of a scalpel each costal cartilage is cut near its junction with the rib carefully, so that underlying pleural membrane is not punctured. The knife should be held as obliquely as possible. The cartilages in elderly are often calcified and are to be cut with bone scissors. First the second rib is cut. After, cutting all cartilages the sternum with its attached cartilages is elevated by carefully dissecting its posterior adhesions specially perichondrium. Later the sternoclavicular joints are dislocated with the help of bone nibbler from the posterior aspect taking care not to injure the subclavian veins. Alternatively cut through the sternum at the angle of Louis and remove the piece of sternum along with costal cartilages. The thoracic cavity is exposed.
4. **Opening of Abdominal Cavity**: After the flap of skin, subcutaneous fat and muscles are raised the peritoneal cavity is opened by a central incision in the peritoneal membrane. Care must be taken not to injure the underlying viscera.
5. **Removal of Viscera**: There are two methods. **First method**, which is the method of choice, is of **enbloc removal of the viscera**. In this method, the knife is directed under the skin in the neck along trachea and larynx to reach the upper end of larynx and then cut across. The oesophagus is tied with gauze as high as possible and cut through above the ligature. The great vessels of the heart are cut as away from the heart as possible. The viscera are dissected from their attachments in thoracic and abdominal cavities, until the lower end of the sigmoid colon is reached. A ligature is tied around the lower end of colon or anal canal and then it is cut below the ligature. The block of viscera is now removed, which consists of larynx, trachea, lungs, heart, oesophagus, stomach, liver, spleen, pancreas, small intestine and large intestine and spread on a dissecting table. In females the uterus is removed with its appendages carefully. A transverse incision is given in the lower third of the uterus and the cavity is examined for signs of conception. If conception is present, the products are removed for detailed examination. In males, the prostate is located and palpated to remove it for examination. The testis is examined in the scrotum and then removed for examination. The kidneys are dissected out taking care not to injure the adrenals. All viscera are placed in anatomical position on the dissecting table. They are examined for gross abnormalities. In the other method, viscera are removed in three blocks other than brain and spinal cord. The method is detailed below.
6. **Cervico-thoracic block**: Heart: The heart is removed first after opening the pericardium. It is essential to palpate the pulmonary arteries before they are opened and also to watch carefully for an embolus when these arteries are incised. It is preferable to examine the heart in a fresh state. The examination begins with the inspection of the coronary arteries. The main right and left coronary arteries left anterior descending and circumflex branches are the vessels most commonly involved. The right coronary artery is opened with a scalpel by an incision on the side of the right atrial appendage. Once opened similar parallel incisions (at distance of 0.5 cm) are continued distally. The artery is examined as far distally as the posterior surface of the right ventricle. The left coronary artery is opened by incision on the side of left atrial appendage and traced distally with dissection of left anterior descending and circumflex branches. Relevant points should be noted as detailed in below. Making a cut across the apex with a bread knife begins the heart dissection. After this cut the dissection of the heart proceeds along the flow of the blood. After opening the right atrium the right ventricle is cut with scissors starting posteriorly along the septum and scissors emerging from the pulmonary trunk anteriorly. The left side is

opened by a cut with a knife along the lateral border which cuts both left atrium and left ventricle, the second cut is along the septum and cuts the anterior wall with the knife passing through the left aortic wall.

7. **Neck Organs**: The skin of the neck is dissected away from the underlying muscles with a long dissecting knife without injuring the skin. An incision is given along the lower border if the mandible from angle to angle, to cut the floor of the mouth. Once the tongue is seen two fingers are inserted above the tongue and it is pulled down to explore the posterior pharyngeal wall. With a scalpel the tongue and neck organs are freed from posterior vertebral attachments and pulled down to remove the block, which includes heart, lungs and oesophagus. The oesophagus is tied just above the diaphragm and cut off. The respiratory system is examined by opening the larynx posteriorly in the midline, cutting the trachea in the midline and then separating both lungs from the trachea by cutting the major bronchi as close to the trachea as possible. The oesophagus and aorta are opened and examined. The thyroid is freed from its covering muscles, incised and examined. The parathyroids are located next in the fatty tissue between the oesophagus and the posterior surface of the lateral lobes of the thyroid. The inferior parathyroids are sometimes situated in the loose connective and fatty tissue extending between the thyroid and the thymic region.
8. **Lungs**: The lungs are weighed and their colour and consistency noted. The dissection of the bronchial tree starts from hilum and the bronchial tree is opened with scissors. The pulmonary vessels are examined by separating the lobes and cutting the vessels by starting in the inter-lobar fissure. Then thin slices of the lungs are cut with a long bread knife along its costal surface.
9. **GIT and hepatobiliary block**: The GIT block includes oesophagus, stomach, duodenum and 1st part of jejunum, liver, gall bladder, pancreas and spleen. It should be removed enbloc. In cases where the gastric contents have to be preserved for chemical analysis the oesophagus should be ligatured before it is cut off. The examination of the gastrointestinal tract follows. The mesentery of the small intestine is lifted and cut through with the knife close to intestinal border after the mesenteric vessels have been examined. After a double ligature has been applied to the uppermost portion of the jejunum it is severed next. Now the jejunum, ileum, caecum with appendix and the entire colon can easily be removed and examined. The duodenum and stomach are opened next and are examined. (In cases where the gastric contents require chemical analysis ligatures are applied at both ends of the stomach to preserve the contents. The pancreas is made visible by pulling the stomach caudally. The papilla of Vater is located and the common bile duct opened. The pancreatic duct is also opened now. The portal ligament is cut through next (watch for the hepatic artery and portal vein) and the stomach with duodenum and pancreas are removed together. The spleen is removed and examined. The vena cava should be located and opened *in situ*. The liver is examined by cutting with bread knife.
10. **Genitourinary block**: Both kidneys attached ureters, urinary bladder, prostate and abdominal aorta are removed enbloc. The kidneys are bisected and the capsule is stripped off. The thickness of the cortex is measured. Any obvious abnormality is noted. The ureters are opened with scissors. The urinary bladder is also opened. (Urine sample is collected before opening with a disposable syringe). In the male, prostate and testis and in the female, uterus with ovaries and tubes are also examined.
11. **Central nervous system**: A wooden block is placed under the shoulders so that the neck is extended as far as possible. The head is now firmly fixed either by an assistant or by a headrest. After inspection of the scalp for possible injuries an incision is made starting from the region of the left mastoid process just behind the ear, extending in a semicircular manner over the parietal bones and ending in the region of the right mastoid process just behind the right ear. The incision should penetrate up to the periosteum. Care should be taken not to destroy any hair. The skin and muscles are now separated from the skull by reflecting the scalp in both directions from the line of incision towards the orbital region, to a line parallel to and about 1 cm above the eyebrow and towards the occipital region to the occipital protuberance. The skullcap is removed next by sawing through the bones. Before removing the calvarium, the proposed saw cuts should be outlined with a sharp instrument in two planes intersecting

at an obtuse angle on the lower lateral portions of the skull. The anterior saw cut should be located just behind the normal hairline. The posterior saw cut is curved inwards at the midline and passes through a point just above the apex of the lambdoidal suture. To avoid sawing through the meninges and brain it is advisable to stop when the saw meets very little resistance. To loosen the skull a chisel and hammer may be used carefully and a large wedge shaped portion of the calvarium is removed. Care should be taken not to soil the hair with blood. The duramater is removed next by making two small incisions with the scissors, one to the right and the other to the left of the mid-line in the region of the frontal lobes. Through each of these incisions one blade of the scissors is introduced into the subdural space and the dura on each side is slit opened on the line along which the skull was removed. The anterior part of the falx is now cut free from the cribriform plate and the dura with the falx is easily separated from the arachonoidea. It is not removed completely but left hanging over the occiput attached to the remainder of the dura that covers the base of the skull and to tentorium. The inner surface of the dura may now be examined. During the process of separation of the dura from the arachnoid the amount and type of fluid found in the subdural space should be noted. The dura and the pia-arachnoid are next inspected for various abnormalities, particularly for an exudate or for any increase in fluid. The basal portions of the pia-arachnoid should be examined after removal of the brain. The brain is now removed. The left hand is inserted between the frontal lobes and the skull and the brain drawn backwards, so that the olfactory and optic nerves are brought into view. The former are left on the brain, the latter are cut through as close to the dura of the skull as possible. While the brain is gradually being drawn back, the internal carotid arteries and the infundibulum of the midbrain are cut through. The temporal lobes are lifted up and the occulomotor nerve and small veins are successively cut close to the base of the skull until the tentorium cerebelli is reached. The tentorium is now slit with the scalpel bilaterally from the posterior clinoid processes along the petrous portions of the temporal bones on both sides. The trigeminal and the other nerve trunks are now carefully cut through. Next, the cervical cord, the first cervical nerve and the vertebral arteries are severed. The brain is now free to be delivered from the cranial vault. The meninges covering the base of the brain, the cisterna magna and the sylvian fissure are now examined and the arteries of the circle of Willis are inspected for any anomalies. The brain is usually examined by means of coronal cross sections cut through the entire brain with a long knife starting from frontal lobe. Each section should be about 0.5 to 1.0 cm thick. Each surface is examined and changes are noted.

12. **Spinal cord**: Examination of the spinal cord is required in patients with central nervous system disorders as well as in cases of trauma. The spinal cord can be removed by the anterior or posterior approach. The anterior approach is preferred. The basic principle is to cut through pedicles to that bodies of vertebrae can be removed.
    a. An essential preliminary step is to free the dura around the foramen magnum from the cranial cavity. To do this the dura around the upper end of the cord is held by toothed forceps and a scalpel blade inserted between the dura and the surrounding bone around the entire circumference of the cord. It is usually possible to do this to a depth of approximately 2 cm.
    b. Access to the spinal column is facilitated by using an initial ‘collar’ incision when opening the body followed by wide removal of the ribs. The lumbar and cervical Para vertebral muscles are dissected free of the vertebra exposing the emerging peripheral nerves of the lumbar and cervical plexuses. Sawing with a fan-tailed blade commences in the lumbar region with the blade placed immediately in front of the emerging nerve roots, and continues up the thoracic region in a line immediately lateral to the rib tubercles, then rostrally to the base of the skull. The angle of the saw blade varies in the different regions of the spine column, being horizontal in the lumbar region, becoming increasingly oblique in the thoracic region and being almost vertical in the cervical region. When sawing, care must be taken not to let the blade plunge too deeply once it has been felt to ‘give’ as it enters the spinal canal, particularly in the cervical region where one is cutting

directly down onto the cervical nerve roots. Finally, an oblique cut is made in the sacrum on either side, allowing the vertebral bodies to be pulled forwards. Adhesions with the underlying dura are freed with a scalpel and the process continued rostrally until all the vertebral bodies are removed and the entire length of the spinal cord exposed.

c. The cord is now removed by dividing the nerve roots of the cauda equina and attaching a pair of Spencer Wells forceps to the dura. The cord is now gently lifted from the spinal canal, freeing any adhesions and cutting through the spinal nerve roots. Representative dorsal root ganglia lying within the intervertebral foramina are easily included with the specimen. In the cervical region, however, further dissection is required to expose the ganglia as here they are more laterally situated. Great care must be taken not to angulate or pull on the cord, as this will cause post-mortem artefactual damage.

d. Once removed, the spinal cord is then fixed prior to further dissection. Ideally it should be suspended in a large tank or drainpipe so that no distortion occurs. A weight is attached to the dura of the lower end to prevent shrinkage of the dura, which may cause buckling of the cord. If no suitable container is available it is acceptable to divide the cord into two or three sections for fixation. The cord is transacted after opening the dura to avoid compression damage.

EXAMINATION OF VISCERA

1 Gross examination.
2 Examination of blood vessels or other ducts for patency.
3 Examination of cut surface for gross abnormalities.
4 Then representative sections are collected for histological examination.

Special examination: In special circumstances, parts of the body, hidden so far have also to be examined. These include eyes, inner ears, pharynx, spinal cord and soft tissues and bones of limbs.

SPECIMEN COLLECTION FOR HISTOLOGICAL EXAMINATION

The specimens should be obtained from representative sites. As a general rule all macroscopically diseased tissue should be taken, including a part of adjacent normal tissue whenever possible. Heart, as a whole should be sent to AFIP. Brain may also be sent as a whole where CNS pathology is suspected. Otherwise representative sections from all parts of brain should be collected. Representative sections should be taken from lungs, liver, spleen, kidney, adrenals, thyroid, breast, prostate, stomach, pancreas and gonads. 10% formal saline should be used in adequate quantity so that tissues are well drowned in it. A small piece of bone marrow is suspended in 1-2 ml of 5% Bovine albumin (one volume 30% albumin, five volumes normal saline). Specimen Containers should:

1. Be wide-mouthed to prevent distortion of specimen.
2. Have a screw top to avoid leakage of the solution used as fixative.
3. Be adequately labelled, including the name, number, rank of deceased with hospital/unit, tissue specimen contained and date of collection.

COLLECTION OF SPECIMENS FOR CHEMICAL ANALYSIS

This is required in brought in dead cases, in death occurring within 24 hours of hospital admission, when chemical poisoning is suspected or when no cause of death is apparent. Collect stomach along with its contents, half of liver, one kidney and a portion of small intestine along with contents tied on each side. Place these in a suitable container containing an excess of saturated solution of sodium chloride. The container is covered with a cloth that is secured with string. Seal the tied string using an impression seal of the unit before forwarding the specimen. The specimen container is labelled, giving full particulars of the deceased and specimens contained with date. These are then sent to the Regional/Provincial Chemical Examiner to Government together with:

1. A sample of the preservative (saturated sodium chloride solution) in a sealed bottle
2. A sample of the seal
3. A copy of the autopsy report

COLLECTION OF SPECIMENS FOR MICROBIOLOGICAL EXAMINATION

All specimens for bacteriological examination e.g., exudates, blood, excreta etc. must be collected using sterile technique and in sterile stoppered containers.

1. **Blood Culture**. Blood for blood culture must be obtained before the organs are disturbed.

When the pericardial sac has been opened the anterior surface of the right ventricle or right atrium is seared with a heated knife and the needle of a sterile syringe is passed into the ventricular or arterial cavity. Blood is withdrawn and injected immediately in an appropriate media container. If the blood is clotted, a clot should be removed taking antiseptic precautions and put in media container.

2. **Meningeal Smears**. The area of meningitis is exposed with the usual sterile precautions. The fluid can also be collected in a sterile syringe from the angle formed between medulla oblongata and foramen magnum.
3. **Splenic Smears**. The spleen is divided and a clean glass slide is placed in contact with the cut surface. This may be required in conditions like malaria and leishmaniasis.
4. **Virological Examination**. For virological examination of brain tissue half of the brain should be fixed in 10% formal saline while the other half should be put in 50% glycerine. If the material can be delivered to the laboratory within 12 hours, no preservation is necessary. The specimen should never be packed in ice.

COLLECTION OF SPECIMEN FOR BIOCHEMICAL EXAMINATION

All specimens for biochemical examination should be collected aseptically, in suitable containers with required preservatives and in sufficient quantity.

Urine: Biochemical examination of urine carried within a few hours of death may be of value. The urine should be collected with a clean syringe from the bladder before it is opened.

Blood: Postmortem blood for glucose, urea etc. is of limited value. In cases of suspected drowning, blood collected from right and left ventricle may be analysed for its sodium chloride or other electrolyte contents. The collection of blood for ethyl alcohol should be taken with the usual sterile syringe with the only precaution that no alcohol is employed and specimen is collected under liquid paraffin layer.

Cerebrospinal Fluid: Biochemical examination of CSF for glucose urea and creatinine may be of value if the fluid is collected within 12 hours of death.

SPECIMENS TO BE COLLECTED IN CASE OF AIR CRAFT ACCIDENTS

Following samples should be taken in case of aircraft accidents.

1. Blood for Glucose in sodium fluoride
2. Blood for lactic acid level in sodium fluoride
3. Blood for alcohol in EDTA under liquid paraffin layer
4. Urine for alcohol under liquid paraffin layer
5. Urine for opiates, cannabinoids and drugs without any preservative
6. Muscle for lactic acid level (if blood can't be taken for this purpose) in saline.
7. Blood for carbon monoxide in EDTA under liquid paraffin

CLOSING OF DEAD BODY

It is our religious, moral and legal duty to prepare the dead body after autopsy so that it resembles the original appearance as closely as possible. When the autopsy is completed and all the necessary specimens are taken, the body is prepared for handing over to the relatives. All the viscera are placed in the body cavity and primary incision is closed by suturing with catgut. The sutures should be close enough to ensure proper closure. Moderate force is applied to tie sutures neither very tense nor very loose. The body is cleaned with cotton soaked in lukewarm water to ensure that no stain of blood or any other body secretion is present. Blood should not be oozing from any point on the body. If there is any it must be closed. The dead body is put on a clean stretcher in supine position and covered with a clean sheet of white cloth. A list is prepared (in triplicate) of the belongings recovered from the dead body e.g., money, ornaments, keys, diary, etc. While handing over the dead body to the relatives, the following certificates should be obtained from them:

1. Handing-taking over certificate of dead body.
2. Handing-taking over certificate of belongings.

Table 58.1: Weights and Measurements of Normal Viscera in Adults

Name of Viscous	Measurement (cm)	Weight (g)	
		Male	Female
Adrenals		6-7	6-7
Brain		1300-1400	1200-1400
Gall bladder	Length 9		
Heart	18x8-9x6	280-350	250-300
Thickness Rt ventricle	3-5 mm		
Thickness Lt ventricle	13-15 mm		
Circumference of valves			
Tricuspid	12		
Mitral	10		
Pulmonary	8.5		
Aortic	7.5		
Kidney	12x6x3	125-170	100-150
Thickness of cortex	12-15 mm		
Liver		1400-1600	1200-1400
Lungs			

Right		625	625
Left		565	565
Ovary			
Reproductive life	4x2.5-3x1.5		
Post menopausal	2x1x0.5		
Pancreas (length)	12x15	60-70	60-70
Parathyroid gland	4-6x2-4x0.5-2 mm	0.3-0.4	0.3-0.4
Parotid gland		15	15
Pituitary gland		0.3-0.6	0.3-0.6
Prostate		20-30	

Spleen	12x7x3	140-170	100-150
Testis	4.5x2.5-3x3	10.5-14	
Thymus			
At birth		13.7	13.7
At 2 years		16.2	16.2
Thyroid gland		20-30	20-30
Uterus			
Reproductive age	8x5x 6		
Myometrial thickness	1.2-1.5		
Post-menopausal	5x3x2		

59. PREPARATION OF MUSEUM SPECIMENS

Museum specimens are precious for teaching and recording medical history. Great care must be taken in their handling, preparation, presentation, labelling and cataloguing. Care of these specimen starts from operation theatre if recovered during operation or from mortuary if removed during autopsy. They should be immediately placed in 10% formalin in such a manner that their appearance and colour is not distorted (see chapter on collection and transport of surgical specimens). These specimens should be dissected intelligently. A sharp knife should be used and clean incisions should be made. If possible sections should be taken from the posterior surface, which is not to be exposed for exhibition. Following are the steps in preparation of museum specimen:

1. **Colour Maintenance**: Kaiserling technique is recommended for colour maintenance. It employs 3 solutions. First the fixation in fluid called Kaiserling fixing fluid. Dissolving 60 g potassium acetate and 30 g potassium nitrate in 400 ml of formalin can easily make it. To it is added 2 litre of tap water. Fixation must be proper and adequate. Always inject fixative into the organ/tissue wherever possible e.g., brain, lungs, limb etc. Blood from specimen is washed with normal saline. Cystic specimen is either injected with fixation fluid or, if already opened, packed with cotton soaked in formalin. Always fix the specimen in a big container to prevent disfigurement and provide adequate time for fixation. The specimen is then transferred to 80% ethyl alcohol and kept in it for 30 min to 4 hours depending upon the size of specimen. Later the specimen is transferred to mounting fluid called Kaiserling mounting fluid. This can be prepared by dissolving 100 g of sodium acetate in 1 litre of water and adding 300 ml glycerine and 5 ml of formalin to it. This makes a crystal clear mounting fluid. If this fluid is cloudy then it should be filtered or 50 ml of saturated solution of camphor in alcohol should be added for each 1 litre of solution.
2. **Mounting**: Museum Jars made of plastic or glass should be used. The jar should be of a size appropriate for the specimen to be mounted. Trimming and dissecting alter the specimen. The exact position of specimen in the jar is decided. Preferably these should be mounted in their anatomical position. If any inside labels are to be used increase the size of jar. Metallic or plastic arrows can be used to highlight specific spots. The presentation of specimen is greatly improved if the specimen is stitched to a central plate. It stabilises the specimen. Holes are drilled into the central plate at appropriate places and the specimen is tied with thread. The central plate is supported at the base of the jar to keep it straight if possible, it is slanted in the jar. Black central plate can be used if a pale colour is to be highlighted. Delicate structures e.g., berry aneurysm at Circle of Willis can be mounted in gelatin with the help of cellotape, which can be removed after the gelatin has set in. The preparation is then placed in the jar. The jar is filled with Kaiserling mounting fluid so that whole of the specimen remains immersed in it.
3. **Labelling**: Labelling a museum specimen is a personal preference. The specimens are always labelled at the bottom in such a manner that it does not obscure the specimen. Label can be placed on the central plate or preferably on the outer surface of the jar (from where it can always be changed or altered). The label consists of specimen number and prefix for the system to which it belongs e.g., FG. for female genital tract. Number of specimen can also be standardised e.g., 01 for congenital lesions 2 for traumatic lesions 03 for benign tumours and 04 for malignant tumours.
4. **Cataloguing**: All specimens in a museum should be catalogued. Preferred method of cataloguing is card system. This provides easy access to a desired specimen. The card should have the specimen number on one corner. The text should consist of detailed information about specimen. Duplicate copies of cards are to be prepared and the other set saved separately.

SECTION IX - NUCLEAR MEDICINE

60. GENERAL ASPECTS

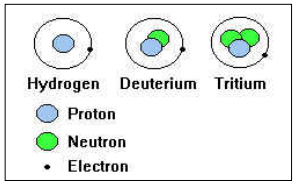
DEFINITION

Nuclear medicine is the branch of medicine concerned with the application of radioactive tracer principles to clinical medicine and biochemical research. The major divisions of nuclear medicine are:

1 Organ imaging e.g., brain scan for the detection of a tumour and whole body imaging e.g., skeletal survey for the detection of metastasis.
2 Organ uptake e.g., determination of thyroid function with radioiodine. Whole body retention, e.g., measurement of the absorption of orally administered vitamin B_{12}. Dynamic studies, e.g., the investigation of renal function, renography; body space, e.g., measurement of plasma volume by isotope dilution analysis.
3 Therapeutic e.g., treatment of hyper-thyroidism and carcinoma of thyroid with radioactive iodine.
4 *In vitro* biochemical analysis, e.g., assay of hormones, enzymes and other substances by radioimmunoassay, saturation analysis and related techniques.
5 All diagnostic procedures performed in Nuclear Medicine are non-invasive. They do not involve any surgical intervention or any manipulation that may be traumatic, painful, or dangerous to life. In all these, isotopes of various elements are employed.

ISOTOPES

Isotopes are one of the several different forms of an element having the same number of protons in their nuclei and therefore the same atomic number. However, they differ in the number of neutrons and hence in the mass number. Isotopes have almost identical chemical properties, as these are a function of the atomic number. The production of artificial radioisotope was achieved as early as 1933. Early isotopes were not only expensive but were not chemically pure to permit their medical use. But the physiologists employed them for the experimental studies of metabolism employed them. The earliest studies performed included the use of radioactive iodine for thyroid function and treatment of thyroid disease (thyrotoxicosis and thyroid carcinoma) and radioactive phosphorus for the treatment of some blood disorders as a substitute for whole body irradiation. It was in 1946 that the radioisotopes became available commercially and at cheaper rates. This promoted their use in all fields of medicine.



FACTORS THAT INFLUENCE USE OF ISOTOPES IN MEDICINE

One might wonder why the use of radioisotopes in medicine has become so important in such a short time. The reason lies in the following facts.

1 The radioactive elements possess the same chemical and biochemical properties as their stable (non-radioactive) forms. In other words the various body processes do not differentiate between stable and radioactive form of elements or compounds labelled with these elements. The thyroid gland concentrates both ^{127}I i.e. the ordinary stable iodine, and ^{131}I (the radioactive iodine) in the same manner without any discrimination whatsoever. Similarly the bone marrow will utilise ^{59}Fe (the radioactive iron) for red cell formation in the same way as it uses the ordinary stable iron.
2 The radiation emitted by the tracer elements (radioactive isotopes) given to the patients can be detected and measured both qualitatively and quantitatively by using special instruments placed outside the body. This gives information about the function of various organs as reflected by the metabolism and fate of the appropriate tracer substance administered. For instance it is possible to assess the function of the kidney by putting a radiation counting instrument over the organ after injecting a tracer dose of a radioactive chemical that can be excreted by the nephrons. All such procedures that involve administration of radioisotopes internally and measurement of radiation in a living person externally to obtain diagnostic information are called *in vivo* procedures.
3 In most of the diagnostic studies the amount of the radioactive substance used is so small that it does not interfere with the normal functions of various organs and does not

have toxic effects. For instance, weight of the radioactive substance used for kidney function test is only a few micrograms. This test can, therefore, be performed even in cases of acute renal failure and will not cause any damage to the organ. There is also no need for doing a sensitivity test.

4 In various specimens obtained from the human body, after giving a radioactive tracer element, it is easier to measure the radioactivity than doing chemical analysis of various ingredients. Thus, radioactive techniques are easy to perform and often give equally valuable or even better information than the chemical analytical procedures available.

5 Various body organs have special affinity for various chemical substances. Such substances can be produced in radioactive form and given internally in order to selectively irradiate these organs. This procedure is used for therapeutic purpose.

6 It is possible to label various pharmaceutical compounds with appropriate radioactive elements and study their absorption, metabolism, and fate in the animals and human beings. For instance to see the rate of absorption of a drug after subcutaneous injection, the drug is labelled and then injected and the rate of its disappearance (absorption) from the site of injection can be measured by using an ordinary radiation counting instrument placed on the skin.

7 Certain radioisotope procedures for diagnostic purpose can be performed on serum or tissue samples and do not involve administration of any radioactive substance to the patient. Such procedures are called *in vitro* procedures. Here the specimens obtained from the patient are made to react or combine with radioactive substances in the test tube (as radioimmunoassay techniques) or they are made radioactive by a neutron source (as in neutron activation analysis) to obtain valuable diagnostic information. Such procedures do not involve any radiation exposure to the patient.

TERMINOLOGY

Atom: It is the smallest particle of an element that is capable of entering into a chemical reaction.

Electron: A negatively charged particle rotating in an orbit around nucleus.

Nucleus: That part of an atom in which most of the mass and the positive electric charges are concentrated.

Proton: An elementary nuclear particle with a positive electric charge. It equals numerically to the charge of the electron.

Neutron: An elementary, electrically neutral nuclear particle with a mass approximately the same as that of a proton.

Atomic number (Z): The number of protons or positive charges in the nucleus. It also reflects the number of electrons outside the nucleus of a neutral atom.

Atomic weight: The relative weight of the atom of an element compared with the weight of one atom of carbon that is taken as 12.

Nuclide: A general term referring to any nucleus, (stable or radioactive) plus its orbital electrons.

Metastable State: An excited state of a nucleus that returns to its ground state, by the emission of γ rays. Ground state is not achieved immediately but is measured in half-life.

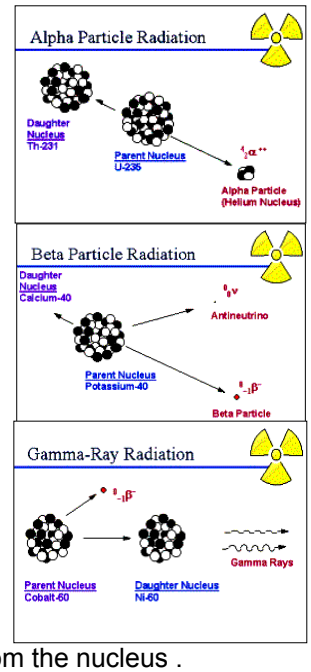
Half life (t½): The time in which half of the radioactive isotope decays.

Radioactivity: The process by which certain nuclides undergo spontaneous disintegration and liberate energy. This generally results in the formation of new nuclides. The process is accompanied by the emission of one or more types of radiation, such as α and β particles and γ radiation.

Becqueral (Bq): The SI unit for radioactive decay 1 Bq=1 disintegration per second.

Millicurie (mCi): It is a unit of radioactive decay equal to $3.7X10^{7}$ disintegration per second. (1 mCi=37.17 MBq).

α-Particle: It is a helium nucleus, consisting of 2 protons and 2 neutrons. It has a double positive charge.

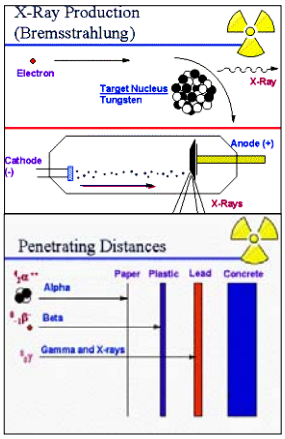


β-Particle: A charged particle emitted from the nucleus of an atom. Its mass and the charge are equal in magnitude to that of an electron.

γ-Rays: A short wavelength, electromagnetic radiation of nuclear origin with a range of wavelength form 10^{-9} to 10^{-12} cm, emitted from the nucleus .

X-Rays: Penetrating electromagnetic radiations

having wavelength much shorter than those of visible light. Penettrating distances of various rays are shown in .



Electron Volt (eV): The amount of energy gained by an electron as it passes through a potential difference of 1 volt.

Absorbed dose: Mean energy imparted by ionising radiation to a small volume of matter, divided by the mass of the matter in kg. Former special unit the rad= 0.01 JKg-1 or 100 ergs-g-1.

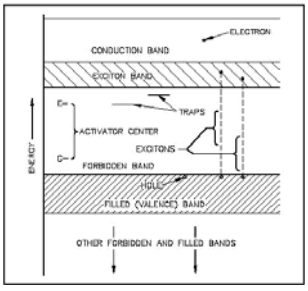
Radiopharmaceuticals: These are medical products, designed for use in the investigation and treatment of human disease and contain a radionuclide as an integral part of the main ingredient. Radiopharmaceuticals differ from conventional pharmaceutical in that:

1 They are radioactive.
2 The mass of the main ingredient that administered is generally too small to produce a pharmacological response.
3 They are usually given to provide useful information.

Radiopharmaceutical Kits: These contain all the non-radioactive ingredients required for the preparation of an injectable radiopharmaceutical in a pre-packed sterile form that has guaranteed quality. These kits are designed for use with short-lived radionuclides obtainable from generators.

Scanner: A device used to display a two-dimensional portrayal of the variations of concentration of radioactivity in any volume of material.

Scintillation Counter: The combination of NaI (Tl) crystal, photomultiplier tube, and associated circuits for counting light emissions produced in the NaI (Tl) crystal by ionising radiation.



Tracer: It is the substance that is labelled with a radionuclide (^{125}I, ^{3}H, ^{14}C) and is used to aid measurement of the unlabelled counterpart.

Scintillation imaging: Examination involving the recording of images that could be carried out either with the aim of studying the morphology and structure of an organ (in which case the distribution of radioactivity in the organ must remain stable throughout an examination lasting several hours) or with the aim of providing dynamic functional information (in which case several successive images have to be recorded). There are two types of examination in this latter category, a slow kinetic phenomena, in which the time required to record an image and the time interval between successive images are not limiting factors, and studies of fast kinetic phenomena, in which the images must be produced quickly in a rapid succession.

Organs Imaging Devices

- Rectilinear imaging device
- Multicrystal whole body scanner
- Multiplane tomographic scanner
- Single crystal imaging device
- γ camera
- Emission computerised tomography
- Positron Emission tomography (PET)

Generators: The idea of generator is that the radionuclide of interest should be the daughter of the long-lived parent radionuclide from which it can be separated easily by physical or chemical means.

MEASUREMENT OF RADIATION

Ionising radiation cannot be seen, felt or sensed by the body and the damage to human tissue is dependent on the energy absorbed by the tissue as a result of ionisation. The term used to describe energy absorption in an appropriate part of the human body is dose. The modern unit of dose is the Gray (Gy). However, in practical radiation protection, in order to take account of certain biological effects, the unit most often used is the Sievert (Sv). For X-ray, γ and β radiation, one Sievert corresponds to one Gray. The most important equipment for the user is a radiation monitoring device. There are instruments and other devices that depend on the response of film or solid-state detectors (for example, the film badge or thermoluminescent dosimeters). Two types of instruments are available: dose rate meters (also called survey meters) and dosimeters. Modern dose rate meters are generally calibrated to read in microsieverts per hour (μSv.h-1). However, many instruments still use the older unit of millirem per hour (mrem.h-1). 10 μSv.h-1 is equivalent to 1 mrem.h-1.

RADIATION DOSIMETRY AND RADIATION PROTECTION

Radiation effects: Radiation carries energy that may damage living cells. The damage may cause cells either to die or to change their structure and function. Over an extended period, the body can repair most of the small damages from almost any cause, including radiation, but if the dose is acute, that is large dose in short period, more serious damage may occur. At lower doses, radiation exposure results in some likelihood of developing cancer and leukaemia and this likelihood decreases in proportion to the dose. Doses resulting from natural background radiation produce a very small fraction of the number of recorded cancer cases. This property of causing cancer is one that radiation shares with a large number of chemicals and other materials, both natural and man-made. Exposure to radiation may also cause genetic defects that could appear in future generations. These effects are divided into two groups:

1 **Stochastic**: Those effects for which probability of an effect occurring rather than its severity is regarded as a function of dose without threshold. An annual dose equivalent limit for radion workers, for uniform irradiation of the whole body is 50 mSv (5 rem).
2 **Non-stochastic**: Effects for which severity varies with dose, e.g., cataract of lens, non-malignant damage to skin, cell depletion in bone marrow. A dose equivalent limit of 0.5 Sv (50 rem) in a year applies to all tissues, except lens of eye, for which the value applied is 0.3 Sv (30 rem).

Protection from ionising radiation: In everyday life we cannot avoid the bulk of natural background radiation. However, protection from man-made sources of radiation can be achieved by increasing distance, reducing time of exposure and by using shielding material. The International Commission on Radiological Protection (ICRP) has recommended annual dose limits for radiation workers and members of the public. These dose limits are so designed as to reduce the probability of any harmful effect during the lifetime of the exposed person.

RADIATION PROTECTION IN PAKISTAN

In 1984, Pakistan Nuclear Safety and Radiation Protection (PNSRP) Ordinance was promulgated and Pakistan Atomic Energy Commission was made responsible for regulating the use of radiation facilities and radiation apparatus within the country. Under the ordinance, the Directorate of Nuclear Safety and Radiation Protection (DNSRP) was created in 1985. Pursuant to the PNSRP Ordinance, the Pakistan Nuclear Safety and Radiation Protection (PNSRP) regulations 1990 have been notified in the Gazette of Pakistan for the control on nuclear establishments and radiation handling facilities, including the use of X-rays, within the country. These regulations are in conformity with the recommendations and guidelines of the ICRP and International Atomic Energy Authority (IAEA).

RULES FOR SAFE USE OF RADIOPHARMA-CEUTICALS

1. Wear overalls/laboratory coats or other protective clothing at all times in areas where radioactive materials are used.
2. Wear disposable gloves at all times while handling radioactive materials.
3. Either after each procedure or before leaving the area monitor your hands for contamination in a low background area with a crystal probe or camera.
4. Use a syringe shield for routine preparation of multi-dose vials and administration of radiopharmaceuticals to patients.
5. Do not eat, drink, smoke or apply cosmetics in any area where radioactive material is stored or used.
6. Do not store food, drink or personal effects in areas where radioactive material is stored or used.
7. Wear personnel monitoring devices at all times while in areas where radioactive materials are used or stored. These devices should be worn as prescribed by the Radiation Safety Officer. When not being worn to monitor occupational exposures, personnel monitoring devices should be stored in the work place in a designated low background area.
8. Wear a finger exposure monitor during the elution of the generator, during the preparation, assay and injection of radiopharmaceuticals and when holding patients during procedures.
9. Dispose off radioactive waste only in designated, labelled and properly shielded receptacles.
10. Never pipette by mouth.
11. Wipe-test all by-product material, storage, preparation and administration areas, weekly, for contamination. If necessary, decontaminate or secure the areas for decay.
12. With a radiation detection survey meter, survey the generator storage, kit preparation

and injection areas daily for contamination. If necessary, decontaminate or secure the area for decay as appropriate.

13. Confine radioactive solutions in shielded containers that are clearly labelled. Radiopharmaceutical multi-dose diagnostic vials and therapy vials should be labelled with the isotope, the name of the compound, and the date and time of the receipt of preparation. A logbook should be used to record the procedural information and total prepared activity, specific activity as Bq/cm^3 at a specified time, total volume prepared, total volume remaining, the measured activity of each patient dosage, and any other appropriate information. Syringes and unit dosages should be labelled with the radiopharmaceutical, type of study, or the patient name.
14. Assay each patient dosage in the dose calibrator before administering it. Do not use a dosage if it is more than 10% off from the prescribed dosage, except for prescribed dosages of less than 10 μCi. When measuring the dosage, you need not consider the radioactivity that adheres to the syringe wall or remains in the needle. Check the patient's name and identification number and the prescribed radionuclide, chemical form and dosage before administering.
15. Always keep flood sources, syringes, waste and other radioactive material in shielded containers.
16. Because even sources with small amounts of radioactivity exhibit a high dose rate on contact, you should use a cart or wheelchair to move flood source waste and other radioactive material.

Special precautions:

1. **Licence**: The laboratory must meet the licensing authorities specifications concerning the use of radioactive materials, where this is legally required.
2. **Ordering and storage**: It is practical to enter every receipt of radioactive materials as well as their disposal in a book specially designated for this purpose. Storage of ^{125}I-labelled compounds, especially in the mCi-range, is best carried out using lead vessels with a 4-5 mm wall thickness. These vessels are best stored in locked safe specially constructed for such purposes. The safe should itself be fire proof.
3. **Waste disposal**: Liquid and waste should be stored separately and should contain only one isotope. The disposal of radioactive waste should follow the local authorities legal requirements. In many countries it is forbidden to dispose of liquid or solid radioactive waste through the normal channels.
4. **Personnel control**: All people regularly working with radioisotopes should wear a film badge. Those who often work with higher concentrations of radioisotopes should in addition, wear a pocket dosimeter or a finger dosimeter.
5. **Testing for contamination**: Laboratory benches should be checked daily with a portable Geiger-Muller counter for contamination caused by spilled reagents. It is also recommended that the laboratory personnel are checked when leaving the area in which radioactive isotopes are used, by checking the radioactivity on hands, clothes and shoes. Decontamination should be carried out by an expert radiophysicist. To prevent contamination of the benches in this way it is recommended that when working with radioactive materials, the bench be covered with a suitable material, i.e., benchkote or a similar material with a non-absorbent surface placed facing upwards and the very absorbent surface placed face downwards on the bench top.

61. RADIOIMMUNOASSAY

INTRODUCTION

It is a test system in which the binding component is an antibody and in which the antigen is labelled with a radioisotope. The radioimmunoassay technique is based on the isotope dilution principle, along with the use of a specific antibody to bind a portion of a substance to be measured. If an antigen (for example, a hormone) is mixed with a specific antibody to that substance, an interaction will occur forming an antigen antibody complex that is chemically different from either the antigen or the antibody. If there is insufficient antibody to complex with the entire antigen present, mixing of the antibody with a known amount of isotopically labelled antigen along with an unknown amount of unlabelled antigen allows quantitation of the unlabelled antigen. The following example may help to illustrate the point. If the amount of antigen present is fixed, then by competitive binding the amount of antigen-antibody complex formed will be inversely dependent on the amount of unlabelled antigen present. Another way of explaining this is that the left over labelled antigen remaining will be in direct proportion to the unlabelled antigen present in the reaction mixture.

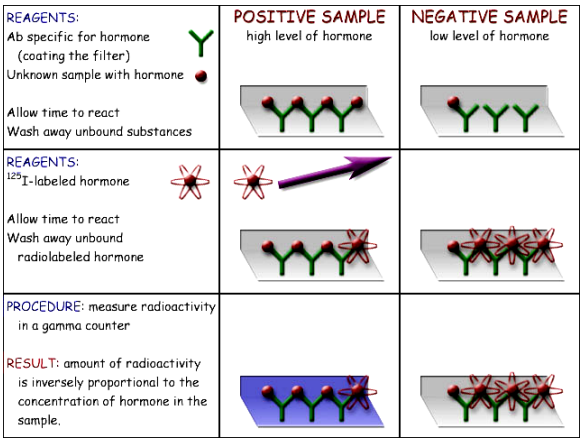


Figure 61.1: Principle of radioimmuno assay (RIA)

COMPONENTS OF THE RADIOIMMUNOASSAY SYSTEM

These are:

1 Equilibration or incubation.
2 A method of separation of bound from free elements.
3 Counting of the radioactivity in the components.

Interpretation of the results requires analysis of the amount of radioactivity in the bound and free forms of the patient's plasma (unknown) relative to concentrations of standards.

REQUIREMENTS

1 A highly purified preparation of the antigen.
2 Radiolabelled antigen.
3 Antiserum with good binding affinity to antigen.
4 A method for the separation of antigen-antibody complex from the free antigen.

The sensitivity of an assay depends to a large extent on the quality of these components and choice of a particular separation technique.

Pure Antigen: The production of antibodies depends on the availability of pure antigen. Several procedures, such as electrophoresis, chromato-electrophoresis, gel filtration, ion exchange chromatography, and precipitation by salt and organic solvents, are available for the extraction and purification of hormones from biologic samples. A pure synthetic preparation if available can be substituted for the natural preparation. A number of hormones produced synthetically are now available with a purity to match the best material isolated from natural sources. In any case the specificity between the antigen and the antigen in the test sample towards the antibody binding sites must be clearly established.

Radiolabelled Antigens: A radiolabelled antigen possessing an unimpaired reactivity with an antibody is one of the essential components of any radioimmunoassay test. The sensitivity of an assay is dependent on two factors relating to the antigen. The labelled antigen should react with the antibody in the same fashion, as does the unlabelled antigen. If a portion of the labelled antigen fails to react with the antibody, then an increased amount of antibody will be required to allow formation of sufficient labelled complex to provide a suitable counting rate.

Antigen Labels: The choice of a radionuclide for labelling purposes is dictated mostly by the availability of a suitable procedure to tag the antigen under study. Radionuclide half-life, specific activity available and cost is to be considered. ^{3}H and ^{14}C find application in steroid

analyses, in which it is convenient to tag these radionuclides into the ring portions of the molecule. The inherent disadvantage in using these isotopes arises from their half-life and pure β-emissions. ^{57}Co has found application only in the Vitamin B_{12} radioimmunoassay. ^{75}Se is also used in limited applications. The most widely used radionuclide in the radioimmunoassay of peptide hormones, viral antigens, and drugs are the isotopes of iodine. The incorporation of iodine into polypeptides and proteins that contain tyrosine residues can be easily achieved. The main advantage of these isotopes is that they can be obtained in higher specific activities than can be found with either ^{3}H or ^{14}C. ^{125}I has become the isotope of choice for most compounds in radioimmunoassay. The reasons for preferring ^{125}I over ^{131}I are many:

1 The isotopic abundance of ^{125}I as supplied by many commercial firms is over 96% whereas the isotopic abundance of a ^{131}I preparation is about 5% to 20%.
2 The counting efficiency for ^{125}I is much higher than that for ^{131}I because of the higher energy of the later.
3 The longer half-life of ^{125}I (60 days) compared with that of ^{131}I (8.06 days) prolongs the shelf life of a particular preparation.
4 The handling of ^{125}I presents a lesser health hazard than the handling of ^{131}I.

Specific antibody: The main criterion for a suitable antibody concerns its specificity for antigen interaction. This specificity is influenced by the heterogeneity of antibodies for the same antigen i.e. cross reactivity for similar substances. Since the antibodies are produced in a variety of animals, species variation may be important. Other influences on the interaction include antigenic damage that occurs with time (as enzymes) and nonspecific binding of other legends.

Standards: These are either identical or similar to the unlabelled antigen (legends) and are most important in that they must also behave like the unknown in the system. Non-identity with the substances occurs because of:

- Natural variations such as big insulin fragments of hormones and other substances.
- Species differences
- Artefacts produced by degradation
- Synthetic errors in the manufacture of artificial substances.

The stability of the antigen used as a standard becomes critical because the standards are often used in assays over a long period of time. There are interfering substances that may cause non-identity of the standards with the unknowns. These include factors such as heparin (and other drugs), urea, bilirubin, buffers, temperature effect and pH.

Patient Sample: This is really the antigen (unlabeled) to be measured in the serum or plasma and is analogous or identical to the unlabelled standard antigen.

The Reaction: The elements described are incubated together to allow a reaction to occur. The competition between labelled and unlabelled legends is allowed to occur simultaneously until equilibrium is reached between the legend and the binder (antibody).

Separation of Bound from Free Components: There are several methods used for separating bound component from the free component. These are often selected arbitrarily. The methods include differential migration (by chromatography, electrophoresis, gel filtration etc), precipitation of the bound form (such as by a double antibody technique) and absorption of free phase by charcoal, resin, polypropylene tubes etc. Other methods include dialysis and ultra-filtration to accomplish the separation.

PROCEDURE

The actual procedure consists of two major steps, the establishment of the assay and the assay of the known sample quantity.

The Assay is established by:

1 Obtaining the components necessary to perform the assay.
2 Allowing the components to interact under the optimal conditions.
3 Separating specific components.
4 Measuring those components by radioactive tracer measuring techniques.
5 Obtaining measurements of a series of known quantities of the substances for later comparison to an unknown amount in the patient sample.
6 The unknown sample quantity can be measured following establishment of the assay and preparation of the standard curve, the patient sample may thus be placed in the solution, representing an unknown amount of the antigen. This unknown amount can then be calculated by knowing the amount of bound radioactively labelled antigen (from the counts obtained in the γ counter) and by interpolation (of these counts) on the standard curve.

The term competitive binding is an appropriate name for the above system of assay.

EQUIPMENT USED FOR RADIOIMMUNOLOGICAL DETERMINATIONS

- Liquid scintillation counter
- Gamma counter [NaI (Tl) detector]

APPENDICES

Appendix I: Biochemical Milk analysis

Biochemical analysis of milk constitute testing of all or some of the following constituents:

- Specific gravity
- Fat contents
- Non fat solids
- Freezing point measurement

The specific gravity is determined by a lactometer, similar to one used for estimation of specific gravity of urine and other fluids.

Non fat solids are calculated by a special slide rule scale called Richmond Scale.

The determination of fat contents of milk (the Gerber method) was originally devised by Dr N Gerber in 1892 and it still remains the official method of fat testing through out the world. The advantages are that it is a quick and simple method. No calibration of any instrument is required and all types of milk can be tested. The surrounding protein coat protects the milk fat in globules. Concentrated sulphuric acid destroys the organic protein and carbohydrate elements, liberating the fat that is dissolved in amyl alcohol and is separated by centrifugation, and finally read on the scale as percent. This procedure is done in special glass tube called Gerber tube or butyrometer (Figure 61.2).

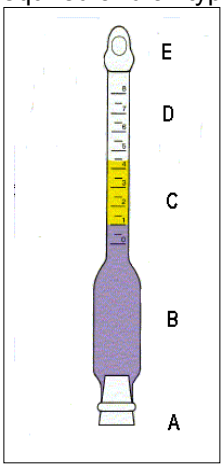


Figure 61.2: Gerber tube (butyrometer) showing A: neck having rubber stopper; B: Body having sulphuric acid-milk mixture; C: Fat column; D: Scale for fat; E: Glass bulb for air.

Procedure

- The Gerber tube is placed in a stand. With a pipette 10 ml concentrated sulphuric acid is placed in the bottom of the tube so that it does not come in contact with the neck.
- Exact 11 ml thoroughly mixed milk is carefully layered over the sulphuric acid without mixing with it and touching the neck of the tube.
- 1 ml amyl alcohol is pipetted on to the milk. Due to lower density, it will form the top-most layer
- Gerber tube is closed with rubber stopper. It is placed in a stand with bulb downwards. The rack is then shaken vigoursly until the two liquids are completely mixed, keeping firm pressure over the stopper with thumb. The tube is inverted up and down several times.
- When the liquids mix, a considerable heat is produced. Therefore, it is suggested to wrap the tube in paper towels or cloth.
- The tube is set vertically in the stand while still hot, stopper pointed downward. It is then centrifuged in Gerber centrifuge, removed form centrifuge and placed stopper down in water bath at 65°C for few minutes.
- The tube is removed form water bath, held vertically and read the % fat from the scale.

Appendix II: Preparation of common buffers

Phosphate buffer, 0.067 M, 5.4-8.2: Prepare 0.067M disodium hydrogen phosphate by dissolving 9.47 g reagent grade anhydrous Na_2HPO_4 in water to make 1L. Prepare a 0.067M solution of potassium dihydrogen phosphate by dissolving 9.08 g reagent grade KH_2PO_4 in water and dilute to 1L. Mix the quantities according to Table 61.1.

Table 61.1: Preparation of phosphate buffer

PH	0.067M Na_2PO_4 (ml)	0.067M KH_2PO_4 (ml)	PH	0.067M Na_2PO_4 (ml)	0.067M KH_2PO_4 (ml)
5.6	5.0	95.0	7.0	61.1	38.9
5.8	7.8	92.22	7.2	71.5	28.5
6.0	12.0	88.0	7.4	80.6	19.4
6.2	18.5	81.5	7.6	87.0	13.0
6.4	26.6	73.4	7.8	91.5	9.5
6.6	37.5	62.5	8.0	94.6	5.4
6.8	49.8	50.2	8.2	97.0	3.0

Tris buffer, 0.05M, *p*H 5.8-9.4: Prepare a 0.1M solution of tris(hydroxymethyl)-aminomethan by dissolving 12.11 g tris in water and diluting to 1L. Prepare a 0.1N solution of HCl. Take 50 ml tris solution in a 100 ml volumetric flask, add the amount of 0.1N HCl as indicated in Table 61.2 and make volume to 100 ml.

Table 61.2: Preparation of Tris buffer

pH	0.1N HCl (ml)	pH	0.1N HCl (ml)
7.2	45.0	8.2	23.3
7.4	42.0	8.4	17.5
7.6	38.9	8.6	12.8
7.8	34.0	8.8	9.0
8.0	29.0	9.0	6.3

Barbital buffer, 0.1M, *p*H 6.8-9.4: Prepare a 0.1M solution of sodium diethylbarbiturate (sodium barbital) by dissolving 20.6 g salt in water and diluting to 1L. Prepare a 0.1N solution of HCl. To prepare the buffer, mix together the amounts of solutions given in Table 61.3.

Table 61.3Table 61.3: Preparation of Barbital buffer

pH	0.1M Na barbital (ml)	0.1N HCl (ml)	pH	0.1M Na barbital (ml)	0.1N HCl (ml)

6.8	52.2	47.8	8.2	76.9	23.1
7.0	53.6	46.4	8.4	82.3	17.7
7.2	55.4	44.6	8.6	87.1	12.9
7.4	58.1	41.9	8.8	90.8	9.2
7.6	61.5	38.5	9.0	93.6	6.4
7.8	66.2	33.8	9.2	95.2	4.8
8.0	71.6	28.4	9.4	97.4	2.6

Appendix III: Strengths of common acids and bases

Grade CP	Formula	Molecular weight	Specific gravity	Percent by weight
Hydrochloric acid	HCl	36.5	1.19	37.0
Sulphuric acid	H_2SO_4	98.1	1.84	96.0
Nitric acid	HNO_3	63.0	1.42	70.0
Phosphoric acid	H_3PO_4	98.0	1.69	85.0
Acetic acid	CH_3COOH	60.0	1.06	99.5
Ammonium hydroxide	NH_4OH	35.0	0.90	56.6

Appendix IV: Dilution of concentrated acids and bases

	Normality (approximate)	Ml/L to make 1.0N solution (approximate)
HCl	12.1	83.0
H_2SO_4	36.0	28.0
HNO_3	15.7	64.0
H_3PO_4	44.0	23.0
CH_3COOH	17.4	57.5
NH_4OH	14.8	67.5

Appendix V: Preparation of standard solution of acids and bases

To prepare approximate 1N solutions of some common acids and bases following amounts of concentrated solutions are required to be diluted or salts to be dissolved in 1L distilled water:

1. **Acids**:
   a. Sulphuric acid: 28 ml
   b. Hydrochloric acid: 83
   c. Nitric acid: 64 ml
   d. Glacial acetic acid: 58 ml
2. **Bases**:
   a. Ammonium hydroxide: 68 ml
   b. Sodium hydroxide: 40 g
   c. Potassium hydroxide: 60 g

Appendix VI: Facilities available at Nuclear Medical Centre, AFIP, Rawalpindi:

- Thallium/MIBI Myocardial perfusion scan
- Radionuclide cardiac angiography (MUGA test)
- Liver scan
- Hepatic radionuclide angiography
- Hepatobiliary scan
- Spleen scan
- Brain scan
- Radionuclide cerebral angiography
- Static bone scanning
- 3-phase bone scanning
- Bone marrow scanning
- Pulmonary perfusion scanning
- Radio-aerosol ventilation imaging
- ^{131}I Hippuran probe renography
- Tc-^{99}m DMSA static renal scan
- Tc-^{99}m DTPA dynamic renal study with Furusemide washout test & GFR estimation
- Vesico-ureteric reflux study
- Bladder residual urine volume estimation
- Renal transplant scintigraphy
- GIT bleeding studies
- Meckle's scan
- Parathyroid scan
- ^{131}I uptake test
- Thyroid scan
- ^{131}I whole body scan
- Salivary gland imaging
- Radionuclide venography
- Testicular scanning
- Lymphatic scintigraphy
- ^{131}I treatment for hyperthyroidism/thyroid carcinoma.
- MIBG Adrenal studies
- Ultrasound organ imaging
- Nuclear Haematology
- ^{51}Cr RBC survival and Sequestration study
- RBC mass estimation
- Plasma volume estimation
- Tc-^{99}m DTPA ventriculography
- Detection of CSF leakage by radionuclides
- SPECT imaging for various organs

Appendix VII: Molecular Genetic Facilities available at AFIP

The following facilities are available:

1. Prenatal diagnosis of inherited disorders: Thalassaemia and other Haemoglobin disorders, Duchenne Muscular Dystrophy, Haemophilia-A, Cystic fibrosis, Foetal sexing (for X-linked disorders only), Foetal Rh-D typing, Down's syndrome (Trisomy 21), Trisomy 13 and Trisomy 18.
2. Neoplastic disorders: Immunoglobulin and T-cell receptor gene rearrangement, Bcl-2 gene rearrangement, Bcr-abl gene rearrangement, and Pml-gene rearrangement.
3. Infectious disorders: Hepatitis C, EBV, Dengue, HIV, Tuberculosis, Salmonella etc.

Appendix VIII: How to despatch samples for DNA analysis

In view of the sensitive nature of the test it is strongly recommended that patients who want to have DNA analysis should report in person to AFIP. However, in special circumstances the samples can be obtained at a different centre and despatched to AFIP for further analysis. In order to make adequate use of the facility the following procedure should be observed:

1. The sample can be entertained only if it is accompanied by complete clinical information including the ethnic group and the parental consanguinity (degree of relatedness of the parents), and any laboratory tests done.
2. From each person 3-5 ml of blood should be collected in a sterilised EDTA container. The sample should reach AFIP within 24-36 hours of the collection. The blood sample can be transported at temperatures between 20-30°C provided all precautions are taken to ensure sterility of the sample collection and the container.
3. If prenatal diagnosis for a genetic disorder is requested the blood samples from both parents and one affected child (if available) should be included. In most cases of thalassaemia prenatal diagnosis is carried out by direct mutation analysis. However, in most of the other inherited disorders and some cases of thalassaemia prenatal diagnosis is carried out by an indirect approach of linkage analysis. In such cases it is absolutely essential that the affected couple must have at least one affected child and the sample from the affected child must accompany the samples from the parents and the foetal sample.
4. An important requirement for prenatal diagnosis is to obtain adequate amount of foetal sample. This is done by Chorionic Villus Sampling (CVS). The procedure can be carried out at any time after 10 weeks of gestation. The CVS done at some other place can be sent to AFIP without deterioration provided it is collected and transported properly. The ideal collection/transport medium is RPMI-1640. Once collected, the sample should reach AFIP within 12-24 hours, preferably packed in ice.

The usual reporting time for DNA analysis including prenatal diagnosis is one week. Any additional information about the test facilities can be obtained directly from AFIP, Rawalpindi (Tel: 561-30508).

Appendix IX: Elements Listed by Atomic Number

Atomic Number	Name	Symbol	Atomic Number	Name	Symbol
1	Hydrogen	H	52	Tellurium	Te
2	Helium	He	53	Iodine	I
3	Lithium	Li	54	Xenon	Xe
4	Beryllium	Be	55	Cesium	Cs
5	Boron	B	56	Barium	Ba
6	Carbon	C	57	Lanthanum	La
7	Nitrogen	N	58	Cerium	Ce
8	Oxygen	O	59	Praseodymium	Pr
9	Fluorine	F	60	Neodymium	Nd
10	Neon	Ne	61	Promethium	Pm
11	Sodium	Na	62	Samarium	Sm
12	Magnesium	Mg	63	Europium	Eu
13	Aluminum	Al	64	Gadolinium	Gd
14	Silicon	Si	65	Terbium	Tb
15	Phosphorus	P	66	Dysprosium	Dy
16	Sulfur	S	67	Holmium	Ho
17	Chlorine	Cl	68	Erbium	Er
18	Argon	Ar	69	Thulium	Tm
19	Potassium	K	70	Ytterbium	Yb
20	Calcium	Ca	71	Lutetium	Lu
21	Scandium	Sc	72	Hafnium	Hf
22	Titanium	Ti	73	Tantalum	Ta
23	Vanadium	V	74	Wolfram	W
24	Chromium	Cr	75	Rhenium	Re
25	Manganese	Mn	76	Osmium	Os
26	Iron	Fe	77	Iridium	Ir
27	Cobalt	Co	78	Platinum	Pt
28	Nickel	Ni	79	Gold	Au
29	Copper	Cu	80	Mercury	Hg
30	Zinc	Zn	81	Thallium	Tl
31	Gallium	Ga	82	Lead	Pb
32	Germanium	Ge	83	Bismuth	Bi
33	Arsenic	As	84	Polonium	Po
34	Selenium	Se	85	Astatine	At
35	Bromine	Br	86	Radon	Rn
36	Krypton	Kr	87	Francium	Fr
37	Rubidium	Rb	88	Radium	Ra
38	Strontium	Sr	89	Actinium	Ac
39	Yttrium	Y	90	Thorium	Th
40	Zirconium	Zr	91	Protactinium	Pa
41	Niobium	Nb	92	Uranium	U
42	Molybdenum	Mo	93	Neptunium	Np
43	Technetium	Tc	94	Plutonium	Pu
44	Ruthenium	Ru	95	Americium	Am
45	Rhodium	Rh	96	Curium	Cm
46	Palladium	Pd	97	Berkelium	Bk
47	Silver	Ag	98	Californium	Cf
48	Cadmium	Cd	99	Einsteinium	Es
49	Indium	In	100	Fermium	Fm
50	Tin	Sn	101	Mendelevium	Md
51	Antimony	Sb	102	Nobelium	No
			103	Lawrencium	Lr

INDEX

PERIODIC CHART OF THE ELEMENTS

IA	IIA	IIIB	IVB	VB	VIB	VIIB	VIII		IB	IIB	IIIA	IVA	VA	VIA	VIIA	INERT GASES
															1 H 1.00797	2 He 4.0026
3 Li 6.939	4 Be 9.0122										5 B 10.811	6 C 12.0112	7 N 14.0067	8 O 15.9994	9 F 18.9984	10 Ne 20.183
11 Na 22.9898	12 Mg 24.312										13 Al 26.9815	14 Si 28.086	15 P 30.9738	16 S 32.064	17 Cl 35.453	18 Ar 39.948
19 K 39.102	20 Ca 40.08	21 Sc 44.956	22 Ti 47.90	23 V 50.942	24 Cr 51.996	25 Mn 54.9380	26 Fe 55.847	27 Co 58.9332	28 Ni 58.71	29 Cu 63.54	30 Zn 65.37	31 Ga 69.72	32 Ge 72.59	33 As 74.9216	34 Se 78.96	35 Br 79.909	36 Kr 83.80
37 Rb 85.47	38 Sr 87.62	39 Y 88.905	40 Zr 91.22	41 Nb 92.906	42 Mo 95.94	43 Tc (99)	44 Ru 101.07	45 Rh 102.905	46 Pd 106.4	47 Ag 107.870	48 Cd 112.40	49 In 114.82	50 Sn 118.69	51 Sb 121.75	52 Te 127.60	53 I 126.904	54 Xe 131.30
55 Cs 132.905	56 Ba 137.34	✳57 La 138.91	72 Hf 178.49	73 Ta 180.948	74 W 183.85	75 Re 186.2	76 Os 190.2	77 Ir 192.2	78 Pt 195.09	79 Au 196.967	80 Hg 200.59	81 Tl 204.37	82 Pb 207.19	83 Bi 208.980	84 Po (210)	85 At (210)	86 Rn (222)
87 Fr (223)	88 Ra (226)	‡89 Ac (227)	104 Rf (261)	105 Db (262)	106 Sg (266)	107 Bh (262)	108 Hs (265)	109 Mt (266)	110 ? (271)	111 ? (272)	112 ? (277)						

Numbers in parenthesis are mass numbers of most stable or most common isotope.

Atomic weights corrected to conform to the 1963 values of the Commission on Atomic Weights.

The group designations used here are the former Chemical Abstract Service numbers.

✳ Lanthanide Series

58 Ce 140.12	59 Pr 140.907	60 Nd 144.24	61 Pm (147)	62 Sm 150.35	63 Eu 151.96	64 Gd 157.25	65 Tb 158.924	66 Dy 162.50	67 Ho 164.930	68 Er 167.26	69 Tm 168.934	70 Yb 173.04	71 Lu 174.97

‡ Actinide Series

90 Th 232.038	91 Pa (231)	92 U 238.03	93 Np (237)	94 Pu (242)	95 Am (243)	96 Cm (247)	97 Bk (247)	98 Cf (249)	99 Es (254)	100 Fm (253)	101 Md (256)	102 No (256)	103 Lr (257)