

**Vocational Higher Secondary  
Education (VHSE)**

**Second Year**

**MEDICAL LABORATORY TECHNOLOGY**  
*Reference Book*



Government of Kerala  
Department of Education

State Council of Educational Research and Training (SCERT),  
KERALA  
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## FOREWORD

Dear Learners,

This book is intended to serve as a ready reference for learners of vocational higher secondary schools. It offers suggested guidelines for the transaction of the concepts highlighted in the course content. It is expected that the learners achieve significant learning outcomes at the end of the course as envisaged in the curriculum if it is followed properly.

In the context of the Right- based approach, quality education has to be ensured for all learners. The learner community of Vocational Higher Secondary Education in Kerala should be empowered by providing them with the best education that strengthens their competences to become innovative entrepreneurs who contribute to the knowledge society. The change of course names, modular approach adopted for the organisation of course content, work-based pedagogy and the outcome focused assessment approach paved the way for achieving the vision of Vocational Higher Secondary Education in Kerala. The revised curriculum helps to equip the learners with multiple skills matching technological advancements and to produce skilled workforce for meeting the demands of the emerging industries and service sectors with national and global orientation. The revised curriculum attempts to enhance knowledge, skills and attitudes by giving higher priority and space for the learners to make discussions in small groups, and activities requiring hands-on experience.

The SCERT appreciates the hard work and sincere co-operation of the contributors of this book that includes subject experts, industrialists and the teachers of Vocational Higher Secondary Schools. The development of this reference book has been a joint venture of the State Council of Educational Research and Training (SCERT) and the Directorate of Vocational Higher Secondary Education.

The SCERT welcomes constructive criticism and creative suggestions for the improvement of the book.

With regards,

**Dr P.A. Fathima**  
Director  
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# CONTENTS

## **PART - A**

- *About the Course* ..... 05
- *Major skills (with sub skills)* ..... 06
- *Syllabus of Module 3 & 4* ..... 07

## **PART - B**

- *Over view-- Module 3* ..... 15
- *Unit 3.1. Laboratory Management*..... 15
- *Unit 3.2. Clinical pathology* ..... 29
- *Unit 3.3 Clinical Biochemistry* ..... 49
- *Extended Activities - Module 3* ..... 81
- *List of Practical - Module 4* ..... 81
- *Over view-- Module 4* ..... 83
- *Unit 4.1. Diagnostic Microbiology* ..... 83
- *Unit 4.2. Histo technology and Cytology* ..... 135
- *Extended Activities - Module 4* ..... 145
- *List of Practical - Module 4* ..... 145
- *References* ..... 148

## ABOUT THE COURSE

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Medical Laboratory Technology is fast developing along with growing population and technological advancement. It is the most sought job titles in the global Health Care System.

Medical Laboratory Technology is a broad area comprising of different disciplines like Clinical Pathology, Hematology, Biochemistry, Bacteriology, Immunology, Virology, Mycology, Parasitology, Histopathology, Cytology, and Cytogenetics & Molecular biology.

In a country like ours, where fast and tremendous technological advancement and population growth happens, the demand and supply of trained man power is not on par. Introduction of a certificate course in Medical Laboratory Technology at higher secondary level is the remedy to this major skill gap in the country.

Medical Laboratory Technology plays a crucial role in the diagnosis of diseases, prognosis and treatment. Apart from the laboratory diagnosis, application of Medical laboratory technology extends to detection of genetic disorders, epidemiology of infection diseases, detection of metabolic disorders and even to answer unraveled questions in forensic medicine.

The course is designed to provide multi skilled competent personnel in the field of medical laboratory technology to meet the increasing demand. On completion of the course students acquire basic skills in the branches of medical laboratory technology which cater to entry level jobs. The course also provides inroads for students to undergo higher education including research in disciplines of laboratory medicine.

The structure of the course is designed in such a way that the first module of First Year Curriculum familiarizes the learners to the basics of Human Anatomy & Physiology and gives an idea about the important units and features of a Diagnostic Laboratory. The topic also envisages the understanding of proper use and handling of common Laboratory Equipment and Glassware. A proper know - how about Blood, the commonest sample of any laboratory is given as part of the First module so that the learner will have a clear idea about the components, composition and collection of blood.

The second module deals with the common Hematological investigations done in a laboratory. The practical and theoretical exposure will make the learners competent in the field. The second module also covers the topic Blood Banking which has attained much relevance nowadays due to the regular need for blood transfusions.

The third module of the curriculum focuses on the effective management of a Laboratory, various analytical methods and recent advances in clinical biochemistry

and in clinical pathology. The unit familiarises students with different instrument used in Clinical biochemistry from the simplest, micropipette to the most advanced fully automatic STAT Analyser.

Fourth module of the course introduces the learners to the fascinating world of microorganisms and familiarises both traditional and recent trends in microbiology to provide a basic knowledge and imparts skill in diagnostic microbiology. It also covers Histo techniques and cytological techniques, so the learner gets a basic idea about the various steps involved in the preparation of tissue for microscopy. This will help in their future studies or career.

The Curriculum also provides introduction to the automated machineries and techniques which can be experienced during the field visits or as part of OJT (On the Job Training). The laboratories as well as PTCs attached to schools provide ambient atmosphere for attaining perfection in performance for the students. The curriculum of VHSE which gives prime importance to practical is further skill enhanced with the scheduled 'On the Job Training Programs conducted in laboratories both on the government as well as private sector. The school curriculum is further enriched with introduction of ICT enabled teaching-learning methodologies as well as learning activities like survey, camps, expo etc.

### **Major Skills**

- Phlebotomy skill
- Skill in Haematological techniques
- Blood Banking Skill
- Laboratory Management Skill
- Skill in Biochemical techniques
- Skill in Clinical Pathological techniques.
- Skill in Microbiological techniques

### **Sub Skills**

- Measurement of BP and Pulse
- Skill in Handling and operation of common laboratory equipments
- Skill in safe handling of various chemicals
- Skill in First aid practice
- Skill in pipetting
- Skill in reagent preparation
- Tissue processing skill in histopathology

## SYLLABUS

### LABORATORY MANAGEMENT, CLINICAL PATHOLOGY & CLINICAL BIOCHEMISTRY

#### Module 3 Unit 1

#### Laboratory Management

40 periods

Unit No.	Unit	Period
3.1.1	<b>Lab safety</b> Introduction Signs and symbols used in a laboratory Handling and storage of chemicals in a laboratory. Laboratory Hazards-Physical, Chemical, Biological, Electrical, Fire, Radiation Laboratory Safety Precautions-Personal Hygiene Fire Extinguishers Biomedical Waste Management First Aid Practice in Laboratory	20
3.1.2	<b>Laboratory Management</b> Introduction Code of Ethics of a laboratory Professional Role of communication in laboratory Organization of a Laboratory Components of a Laboratory Lay out plan of a multi-room laboratory Organizational pattern of a Laboratory Familiarization of Request forms and report forms. Ordering and Utilization of supplies Maintenance of Stock Registers- Consumables, Non-consumables Accreditation and Certification of Laboratories. Accrediting Agencies- NABL, ISO, CAP, CRISIL - Bar coding and Total Laboratory Automation (TLA) Familiarization of Common Laboratory Software	20

**Module 3 Unit 2****Clinical Pathology****100 periods**

Unit No.	Unit	Period
3.2.1	<b>Introduction</b> Importance, Common specimens, General guidelines for sample collection	10
3.2.2	<b>Urine Analysis</b> <ul style="list-style-type: none"> <li>- Importance, Types of urine samples</li> <li>Methods of collection, preservatives</li> <li>- Physical Examination</li> <li>- Chemical Examination-Sugar, Protein, Blood, Ketone bodies, Bile pigments, Bile salts, Urobilinogen</li> <li>- Microscopic Examination</li> <li>- hCG test in Urine</li> </ul>	44
3.2.3	<b>Sputum Examination</b> <ul style="list-style-type: none"> <li>- Importance, Specimen collection</li> <li>- Physical examination</li> <li>- Microscopic examination</li> </ul>	5
3.2.4	<b>Stool Analysis</b> <ul style="list-style-type: none"> <li>- Importance, Specimen collection</li> <li>- Physical examination</li> <li>- Chemical examination- Occult blood, Reducing substances</li> <li>- Microscopic examination- Saline &amp; Iodine mount</li> </ul>	15
3.2.5	<b>Semen Analysis</b> <ul style="list-style-type: none"> <li>- Importance, Specimen Collection</li> <li>- Physical Examination, Liquefaction Time,</li> <li>- Microscopy- Total Sperm Count, Motility, Morphology</li> <li>- Chemical Examination-Fructose, Acid phosphatase</li> </ul>	16
3.2.5	<b>CSF and other body fluids</b> <ul style="list-style-type: none"> <li>- CSF- Introduction</li> <li>- Specimen collection</li> <li>- Physical &amp; Microscopic Examination</li> <li>- Chemical Examination- protein, glucose ,chloride (Name of method of estimation &amp; clinical significance only)</li> <li>- Other body fluids</li> <li>- Recent advances in Clinical pathology</li> </ul>	10

**Module 3 Unit 3**

**Clinical Biochemistry**

**200 periods**

Unit No.	Unit	Period
3.3.1	<b>Introduction to Biochemistry</b> <ul style="list-style-type: none"> <li>- Types of chemicals and preparation of solutions.</li> <li>- Types of specimens in clinical Biochemistry</li> <li>- Collection and processing of specimens for biochemical analysis</li> <li>- Types of assays- Endpoint and Kinetic (definition and example only)</li> <li>- Cleaning of glass wares for biochemical analysis</li> </ul>	12
3.3.2	<b>Instruments used in Biochemistry</b> <ul style="list-style-type: none"> <li>- Familiarise with Colorimeter, Spectrophotometer, Flame photometer, Centrifuge, Electronic balance, Distillation apparatus, Deionizer</li> </ul>	8
3.3.3	<b>Blood Glucose Estimation</b> <ul style="list-style-type: none"> <li>- Introduction to Diabetes - features, types, complications,</li> <li>- Types of samples- FBS, PPBS, RBS, Anticoagulant used</li> <li>- Methods of estimation- GOD-POD in detail</li> <li>- Normal value and Clinical Significance - Hyper and hypoglycaemia</li> <li>- Mention Glucometer Technique</li> <li>- GTT and GCT procedures,</li> <li>- Mention relevance HbA1C</li> </ul>	28
3.3.4	<b>Renal Function Tests</b> <ul style="list-style-type: none"> <li>- Introduction, Common tests included</li> <li>• Estimation of Blood Urea Mention common methods Urea-Berthelot method in detail, Normal value and Clinical significance Renal, Pre-renal, Post renal conditions of Uraemia</li> <li>• Estimation of S. Creatinine. Mention common methods. Jaffe's method in details, Normal value and Clinical significance</li> <li>• Estimation of Uric Acid. Mention common methods. Uricase method in detail. Normal value and Clinical Significance.</li> <li>- Mention Clearance tests- Urea and Creatinine</li> <li>- Mention Importance of Micro-albumin and Cystatin-C</li> </ul>	40
3.3.5	<b>Liver Function Tests</b> <ul style="list-style-type: none"> <li>• Introduction, Common tests included Bilirubin-Formation of Bilirubin</li> </ul>	40

Unit No.	Unit	Period
	Types of Bilirubin- conjugated and unconjugated Estimation of Bilirubin. Malloy- Evelyn method in detail. Normal value and Clinical Significance <ul style="list-style-type: none"> <li>• Estimation of Total protein- Biuret method in details</li> <li>• Estimation of Albumin- BCG method in details</li> </ul> Normal value and clinical significance of total protein and Albumin, A-G Ratio. <ul style="list-style-type: none"> <li>• Other LFT Parameters- ALP, ALT, AST in brief.</li> </ul>	
3.3.6	<b>Lipid Profile</b> <ul style="list-style-type: none"> <li>• Introduction - Relevance, tests included in the Profile</li> <li>• Estimation of S.Cholesterol. Mention common methods, CHOD-PAP method in detail, Normal value and Clinical Significance Mention Triglycerides, HDL, LDL</li> </ul>	25
3.3.7	<b>Other parameters of Diagnostic importance</b> <ul style="list-style-type: none"> <li>• Serum Electrolytes- Serum Sodium and Potassium Normal value and Clinical significance</li> <li>• Clinically important Minerals- Calcium and Phosphorus (normal value and significance only)</li> <li>• Name Diagnostically important Hormones T3, T4, TSH, FSH, LH, Prolactin, progesterone</li> <li>• Name Clinically important enzymes- Acid Phosphatase, S. Amylase, GGT,</li> <li>• Name Cardiac markers- Troponin-I, Troponin-T CPK, CK-MB, LDH, SGOT</li> <li>• Name Tumour Markers- CA-125, CEA, AFP,CA-19.9, PSA, Beta hCG</li> </ul>	21
3.3.8	<b>Quality control in Biochemistry</b> <ul style="list-style-type: none"> <li>- Introduction, Common terms used in Quality control, Errors - random and systemic , L.J. Chart, External QC and Internal QC</li> </ul>	10
3.3.9	<b>Automation and Recent advances</b> <ul style="list-style-type: none"> <li>Need for Automation, Advantages of Automation Types of Auto Analysers-Semi and Fully automated Electrolyte Analyser (ISE) in brief Advanced Diagnostic Methods in brief</li> <li>- C.L.I.A.,C.L.F.A, Turbidometry, Nephelometry, HPLC, Mention Point of care testing (POCT)</li> </ul>	16

**Module 4 Unit 1**

**Diagnostic Microbiology**

**290 periods**

Unit No.	Unit	Period
4.1.1	<b>Introduction to Microbiology</b> <ul style="list-style-type: none"> <li>• Classification of Microbes, pathogen, commensals, type of Infections, communicable diseases, Carriers Historical aspects in Microbiology</li> </ul>	15
4.1.2	<b>Structure and classification of bacteria</b> <ul style="list-style-type: none"> <li>• Structure- Cell wall, flagella, fimbriae, capsule, spore, plasmid</li> <li>• Classification of bacteria based on morphology- Arrangement, Motility and oxygen requirement</li> </ul>	15
4.1.3	<b>Sterilization and disinfection</b> <ul style="list-style-type: none"> <li>• Importance of sterilization and Disinfection</li> <li>• Methods of sterilization Physical methods- Dry heat, Moist Heat Chemical methods- alcohols, aldehydes, gases Mechanical methods- Filtration, Radiation</li> <li>• Describe principle, parts, and use of - Hot air Oven, Autoclave</li> <li>• Disinfectants and Antiseptics and their application</li> </ul>	45
4.1.4	<b>Growth &amp; Cultivation of Bacteria</b> <ul style="list-style-type: none"> <li>• Bacterial growth and replication - Mention essential growth requirements- Temperature, PH, Gaseous requirements</li> <li>• Culture media Classification of culture media with examples</li> <li>• Preparation and use of common media Peptone water, Nutrient Agar, Blood Agar, Chocolate agar, Mac Conkey Agar</li> <li>• Bacteriological wire loop, Straight wire - Inoculation of Culture media- Liquid and Solid</li> <li>• Mention Streak, Stroke, Stab, Lawn culture - Mention Anaerobic techniques- Gaspak</li> </ul>	40
4.1.5	<b>Basic identification Techniques</b> Introduction Identification of bacteria <ul style="list-style-type: none"> <li>• Different methods</li> <li>• Detection of motility - Name different methods - Hanging drop method in detail</li> <li>• Staining</li> </ul>	50

Unit No.	Unit	Period
	<ul style="list-style-type: none"> <li>- Principle, requirement, procedure and interpretation of Simple stain, Grams stain, AFB stain-Diagnostic significance</li> </ul> Biochemical tests- Coagulase, Catalase, IMViC	
4.1.6	<b>Immunology and its diagnostic applications</b> Introduction <ul style="list-style-type: none"> <li>- Types of Immunity, Antigen ,Antibody</li> <li>- Structure of antibody</li> </ul> Types of antibody- Ig G, IgM, IgA, IgD, Ig E <ul style="list-style-type: none"> <li>• Antigen Antibody reactions- Specificity, Sensitivity, Avidity, Pro-zone ,post-zone, Titre</li> </ul> Clinical applications of Agglutination, precipitation, flocculation, ELISA, Immuno-Fluorescence.	35
4.1.7	<b>Laboratory Diagnosis of Common Bacterial diseases</b> <ul style="list-style-type: none"> <li>• Collection, Processing and transportation of common specimens-Urine, Blood, Sputum, CSF, Stool, Pus, body fluids, swabs</li> </ul> General considerations- Macroscopy , Microscopy, Culture <ul style="list-style-type: none"> <li>• Mention common culture media and identification methods used.</li> </ul> Antibiotic Sensitivity Testing (ABST)- Kirby Bauer Method <ul style="list-style-type: none"> <li>• Common Disease and pathogens encountered -Typhoid, Tuberculosis, Cholera, Dysentery, Syphilis, Leptospirosis, Tetanus, Meningitis&amp; UTI</li> <li>• Common Serological Techniques for diagnosis of Bacterial diseases-</li> <li>• ELISA &amp; its commercial preparations - Immunochromatographic technique</li> <li>• WIDAL,RPR,-Procedure and interpretation</li> </ul>	30
4.1.8	<b>Laboratory Diagnosis of Common Viral diseases</b> <ul style="list-style-type: none"> <li>• Introduction to viruses</li> <li>• Common viral diseases and pathogens encountered - AIDS, Hepatitis, Dengue, Chickun Guinia, Rabies, Infuenza, Mumps and Measles.</li> <li>• Diagnostic techniques for viral infections               <ul style="list-style-type: none"> <li>- Mention common Serological tests used, Latex agglutination, Card tests, ELISA, Tissue culture, PCR Technique</li> </ul> </li> </ul>	20
4.1.9	<b>Laboratory Diagnosis of Common Parasitic diseases</b> <ul style="list-style-type: none"> <li>• Introduction to parasites</li> <li>- Parasite, Commensal, Symbiosis, Host (Intermediate &amp;</li> </ul>	40

Unit No.	Unit	Period
	<p>Definitive host), Vector, Zoonosis</p> <ul style="list-style-type: none"> <li>• Classification-Intestinal &amp; Blood Parasites</li> <li>• Common blood parasites and their lab diagnosis</li> </ul> <ul style="list-style-type: none"> <li>- Blood collection-</li> <li>- Time of collection</li> <li>- Preparation of smear-Thick and thin</li> <li>- Dehaemoglobinisation of thick smear</li> </ul>	
	<p><b>Lab Diagnosis of Malaria</b></p> <ul style="list-style-type: none"> <li>- Disease, mode of transmission, hosts causative agent, types of malaria.</li> <li>- Examination of thick and thin smear-Morphological identification of different stages of parasite</li> <li>- Other stains used- JSB</li> <li>- Other methods- Card method , QBC</li> </ul>	
	<p><b>Lab Diagnosis of Filariasis-</b></p> <ul style="list-style-type: none"> <li>- Disease, mode of transmission, host, and nocturnal habit</li> <li>- Lab diagnosis- wet smear examination, thick smear examination, Concentration technique.</li> </ul>	
	<p><b>Lab Diagnosis of Intestinal parasites</b></p> <ul style="list-style-type: none"> <li>- Introduction -Helminthic infections and parasites</li> <li>- Amoebiasis -Entamoeba histolytica- Disease, Mode of Transmission, Trophozoite &amp; Cyst</li> <li>Lab diagnosis -Macroscopic examination</li> <li>Microscopic examination -Stained &amp; Unstained preparation</li> <li>• Common Helminths- Tape worm, Round worm, Hook worm, Whip worm, Pin worm,</li> <li>- Lab diagnosis-Macroscopic &amp; Microscopic examination</li> <li>- Concentration Techniques of Stool sample- Mention Floatation &amp; Sedimentation methods</li> </ul>	

**Module 4 Unit 2****Histotechnology & Cytology****50 periods**

Unit No.	Unit	Period
4.2.1	<b>Histotechnology</b> Introduction - Methods of examination of Tissues and cells - Gross examination - Microscopic examination • Examination of Unfixed Tissue • Examination of Fixed Tissue - Collection of specimens - Biopsy - Autopsy - Fixation - 10% Formalin - Decalcification	20
4.2.2	<b>Tissue Processing</b> • Steps in tissue processing - Dehydration - Clearing - Impregnation - Embedding Microtomes-Rotary Microtome,-Cryostat - Section cutting - Mention role of adhesives - Staining -H&E Staining - Mounting of Tissue sections - Filing and storage of tissue sections	20
4.2.3	<b>Diagnostic cytology</b> • Introduction • Types of specimens • Processing • Fixation • Staining Advantages and applications in diagnostic cytology	10

## **PART B**

### **Laboratory Management, Clinical Pathology & Clinical Biochemistry**

#### **Module 3**

##### **Over view**

The third module comprises of three vital areas of laboratory medicine, "Laboratory management, Clinical pathology and Clinical Biochemistry". Basic knowledge on laboratory management concepts helps a technician to uphold his professional quality and skill. Clinical Pathology laboratory pertains to analysis of body fluids which enables to reveal pathophysiological maladies and it receives more than 60% of specimens sent for investigations to a diagnostic laboratory. Clinical pathology Techniques are of historical importance in the evolution of medicine and are the cheapest methods with little discomfort and stress for specimen collection providing information of immense value. With the supporting and supplementing information and investigations, the clinicians still largely depend on clinical pathology to resolve diagnostic dilemma.

Clinical biochemistry is the significant part of laboratory diagnosis as it helps to understand biochemical mechanism of the body in relation to diseases. Manual methods and principles of clinical chemistry have been replaced by automated techniques and recent methodologies. The scope of investigations has almost changed the diagnostic scenario from a level of general health assessment to organ function tests.

#### **3.1 Laboratory Management**

The effective operation of a medical laboratory and proper delivery of laboratory results to clinician and their patients are integral part of a well defined health care system. An effective laboratory management is essential for providing an accurate, reliable and timely laboratory results which forms the basis of almost all of the medical decisions and diagnosis made in the modern era. The task of laboratory management involves integration and co ordination of organizational resources like personal, equipment, money, time and space so that standardized planning organization and operation of a laboratory happens. It essentiates management skills in ensuring laboratory safety, handling of laboratory wastes and observing laboratory ethics,

protocols, accreditation and certification criteria. The unit familiarizes the learner with the Code of Ethics of Laboratory professional, safety measures to be taken in a laboratory, tips for personal hygiene and about the care and handling of chemicals in a laboratory. It also creates awareness about the different signs and symbols used in a laboratory, different types of hazards and about the segregation and management of laboratory waste.

Software based laboratory management systems have been evolved over years that support laboratory operations. Most of them utilize web enabled sample management, data analyzing and reporting facilities. Introduction of Bar coding and Total Laboratory Automation has caused tremendous improvement in the patient identification and time management mechanism.

### **Learning outcomes**

#### **The learner:**

- explains different laboratory safety precautions and first aid
- classifies different methods of biomedical waste management
- identifies Code of laboratory ethics and safe laboratory practice
- explains organization of a laboratory, its organizational pattern and the role of Communication in a laboratory
- identifies and prepare lay out of a Medical Laboratory
- formats various request forms, stock registers and order form
- identifies the importance of accreditation, certification of laboratories and identify different accrediting agencies
- identifies the importance of barcoding and Total laboratory Automation and use of Common Laboratory Software

### **3.1.1 Lab safety**

#### **Introduction**

Laboratory safety measures are designed to encourage and promote safe and efficient working practices in a lab. It protects all laboratory personnel and other people with right of entry from illness or injury. All laboratory personnel have responsibility to adhere to and observe safety programme all the time.

Occupational injury and illness are caused mostly by bad practices, inexperience, ignorance and failure to follow standard practices. Safe laboratory Practice mainly depends on,

1. Awareness on Signs & Symbols used in a laboratory
2. Identification of the possibilities for common laboratory hazards
3. Proper management of Bio medical wastes
4. First Aid practices

### Signs and symbols used in a laboratory

To minimize accidents in the laboratory it has been made mandatory to use signs and symbols in the laboratory. These are used to indicate possible hazard during a procedure. A thorough knowledge on signs and symbols helps the laboratory personnel to deliver his duty properly. The symbols include hazard warning symbols, safety (mandatory) symbols and prohibitory symbols. Hazard warning symbols are black pictures in yellow or orange background. Safety symbols are round white pictures with blue background, prohibitory symbols are round black pictures in white background with a red edge and diagonal line.



### Handling and storage of chemicals in a laboratory

Proper handling and storage of chemicals and reagents is necessary to prevent hazards. Chemicals are stored according to their physical and chemical properties

#### Types of chemicals

1. Flammable Chemicals - eg :- Acetone, Ether, Xylene, Alcohol
2. Corrosive chemicals - eg :- Concentrated Acids, Alkalies, phenol etc
3. Oxidising Chemicals - eg :- Potassium dichromate, Chromic acid, Chlorites etc.
4. Explosive Chemicals - eg :- Picric Acid.

5. Radioactive chemicals- eg :- I<sup>125</sup> , I<sup>131</sup> , H<sup>3</sup>
6. Carcinogenic chemicals-eg :- Benzidine, O- toluidine, Selenite, etc
7. Toxic Chemicals -eg :- Potassium Cyanide

### **Handling and Storage of chemicals**

1. Label the chemicals with hazard symbol, simple instruction for use, strength and Expiry date (it is mandatory for manufacturers).
2. Flammable chemicals are stored in fire proof metal containers at ground level in a cool dry place and always handle them away from direct flame.
3. Corrosive chemicals are stored in amber coloured bottles at ground level.
4. Explosive chemicals are never left in dry state.
5. Radioactive chemicals are handled by properly trained persons only
6. Carcinogenic chemicals are stored in closed container. Exposure to such chemicals should be kept minimum.
7. Highly toxic chemicals like Potassium cyanide are kept locked in cupboards and proper documentation is made in stock register.
8. Do not open flammable chemicals near flame
9. Do not add water to acid
10. Avoid mouth pipetting.

### **Laboratory Hazards**

Laboratory hazards are mainly Physical, Chemical, Biological, Electrical, Fire, Radiation hazards.

Physical hazards include Cutting with broken glassware, sharps, Electric shocks, Falling on wet floor, Burns and scald. Burns usually caused by flammable chemicals, faulty electric equipment, Burners, corrosive chemicals, and hot dry objects. Scalds are caused by hot liquids. General precautions to prevent physical hazard in a laboratory are,

1. Proper storage and handling of glassware
2. Adopt Standard electrification methods
3. Proper storage and handling of chemicals
4. Proper handling of lab equipment,
5. Prevent spillage of hot fluids
6. Use quartz chips to prevent violent boiling of liquids

7. Use thick walled glass containers and round bottomed flask for heating purpose
8. Use hazard symbols

Chemical hazards are caused by physical contact, ingestion and inhalation of chemicals, leads to from minor burns to even life threatening. General precautions to prevent chemical hazard in a laboratory are

1. Awareness on the physical and chemical properties of laboratory chemicals
2. Proper storage of chemicals
3. Mouth pipetting should be avoided
4. Dangerous chemicals (toxic chemicals) are kept in small amount for routine use
5. Use hazard symbols

Biological hazards can be caused by infectious agents like Human immunodeficiency virus (HIV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Mycobacterium tuberculosis, Herpes simplex virus etc.

Contaminated food and water, Needle pricks and cuts, Laboratory animal bite can also cause bio hazard. General precautions to prevent Biological hazard in a laboratory are

1. Consider all specimens in the laboratory as potentially dangerous
2. Use gloves, masks, apron and eye protectors in the laboratory
3. Proper waste disposal or management
4. Vaccinate laboratory staff against infectious diseases like HBV
5. Mouth pipetting should be avoided
6. Do not eat, drink, smoke or apply cosmetics in the laboratory
7. Proper washing of hands after work, Cleaning and disinfection of work bench on completion of work

Electrical hazards are mainly caused by faulty operation and improper maintenance of electrical equipment. It may result in minor burns to severe injuries that may lead to death. General precautions to prevent electrical hazard in a laboratory are,

1. All Electrical equipment should be properly grounded
2. Overload circuits should be avoided, do not use extension cords
3. Electrical equipment should not be handled with wet hands
4. Do not disable any electrical safety features.

5. Repair or services should be done by authorized persons only
6. Do not use Equipment for a task not designed for it
7. Do not leave equipment switched on when not in use

Fire in the laboratory may occur due to naked flames, electrical overloading, flammable reagents and smoking in the laboratory. General precautions to prevent Electrical hazard in a laboratory are,

1. Instead of open flames use hot plates
2. Store flammable and explosive chemicals properly
3. Install fire extinguisher in the laboratory. All lab personnel should know the location of fire extinguisher and how to use it
4. In case of fire, escape through fire exit route

### Fire Extinguishers

A fire extinguisher is a fire protection device used to control small fires in emergency situations. Typically, a fire extinguisher consists of a hand-held cylindrical pressure vessel containing agents which can be discharged to extinguish a fire. Different type of fire extinguishers are now available for managing different classes of fires. Fire extinguishers for ABC type fires are commonly used in the laboratories.



**Radiation Hazards** are caused by improper handling of radioactive chemicals. Various analytical procedures like RIA (Radio immunoassay) demand use of radioactive substances in a laboratory. X ray units and other nuclear medicine procedures are also cause radiation hazard. General precautions to prevent radiation hazard in a laboratory are,

1. Radioactive material should be stored in lead shielded container properly labeled with identity.
2. Only trained staff is permitted to handle radioactive chemicals
3. Use hazard symbols
4. The staff should wear safety spectacles, disposable gloves and personal dosimeters.
5. Accurate records are maintained for use and disposal of radio active chemicals.

### Laboratory Safety Precautions-Personal Hygiene

1. Wear gloves and apron
2. Use eye protector if necessary
3. Do not Eat, drink, apply cosmetics inside laboratory
4. Wear shoe/ chappal inside the laboratory
5. Avoid Mouth pipetting.
6. Wash hands properly before and after performing laboratory experiment
7. Disinfect the work bench before leaving the laboratory.
8. Girls must put-up hair inside the laboratory
9. Carry minimum personal articles inside laboratory.

### First Aid Practice in Laboratory.

First aid is the help given immediately to an injured person. For example First aid helps an injured to avoid excessive bleeding from an injury until medical attention has been obtained. A quick and confident approach of first aider can save life from many emergency conditions. Common first aid procedures are given below

**Injuries caused by broken glass:** Wash the wound immediately to remove any glass pieces. Apply mercurchrome or acriflavine ointment to the wound. Cover with gauze and adhesive tape

**Acid/Alkali splashes on the skin:** Wash thoroughly; bath the affected skin with cotton wool soaked in 5% aqueous sodium carbonate if acid and 5% acetic acid or undiluted vinegar, if alkali.

**Acid/Alkali splashes in the eye:** Water spray from a wash bottle or rubber bulb into the medial corner of the eye . Put 4 drops of 2% Aqueous Sodium bicarbonate into the eye, if acid, and saturated solution of boric acid, if alkali.

**Swallowing acid:** Make the patient drink some 5% soap solution immediately. Make him gargle with the soap solution. Give him 3 or 4 glasses of ordinary water. If the lips and tongue are burned by the acid, rinse thoroughly with water. Bathe with 2% aqueous sodium bicarbonate.

**Swallowing alkalies:** Make the patient drink 5% solution of acetic acid or lemon juice or dilute vinegar. Make him gargle with the same acid solution. Give him 3 or 4 glasses of ordinary water. If the lips and tongue are burned by the alkali, rinse thoroughly with water; bathe with 5% acetic acid.

**Poisoning** :-Send for a physician or qualified nurse, specifying the toxic substance involved. Place the victim in open air while waiting for the physician.

**Minor burns:** Plunge the affected part in cold water or ice-water to soothe the pain. Apply Mercurchrome or Acriflavine ointment to the burn. Apply dry gauze dressing loosely. If the burn becomes infected or does not heal, refer the patient to a physician. Never tear off the blisters that form over the burns.

**Severe burns:** If the victim is on fire, roll him in a blanket or overall to smoothen the flames. Inform the physician. Lay the victim on the ground. Do not remove his clothing. Cover him if he is cold. Do not apply any treatment to the burns. This must be left to the physician.

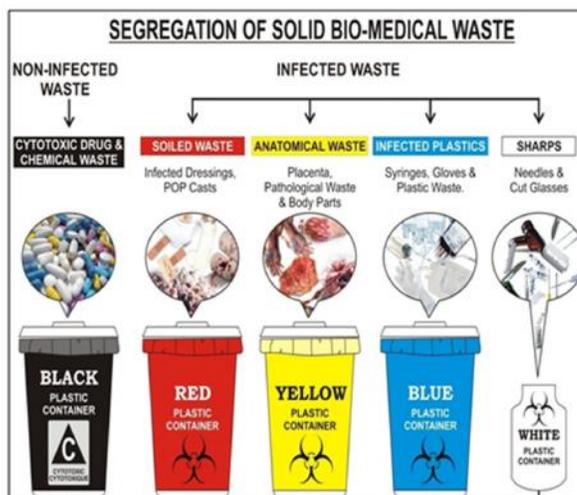
**Unconsciousnes:** If the victim is breathing then lay him face down with his head on one side and arm and the leg of that side in the bent position. This posture makes breathing easier and provides better blood circulation to all body parts. If the victim is not breathing start artificial respiration immediately.

**Artificial respiration:** The procedure should be started quickly as brain infarction occurs within few minutes of oxygen deficiency. Steps of artificial respiration is

1. Lay victim on back
2. Clear any obstruction of the mouth
3. Place an object under the shoulder so the head is tilted back
4. Pinch the nostrils and apply mouth to mouth resuscitation 10 times a minute until breathing starts.

### Biomedical Waste Management in Laboratory

Biomedical waste is generated during the diagnosis, testing, treatment, research or production of bio materials. Management of biological wastes consists of the collection, segregation and proper treatment of individual type of waste. The wastes are colour coded according to the nature of waste and their method of disposal and



segregated at source itself. It is mandatory for each laboratory to adopt proper waste management systems of their own or to be associated with some agencies which do the work in a corporate manner.

### 3.1.2 Laboratory Management

#### **Code of Ethics of a laboratory Professional**

The laboratory personnel should be aware of the code of ethics which is the minimum standard about his professional skill. It includes the do's and don't's in the laboratory. It is a set of principles of right conduct.

1. Treat patients and colleagues with respect, care and thoughtfulness
2. Perform duties in an accurate, precise, timely and responsible manner
3. Safeguard patient information as confidential, within the limits of the law
4. Prudent use of laboratory resources
5. Advocate the delivery of quality laboratory services in a cost effective manner
6. Work within the boundaries of laws and regulations
7. Strive to improve professional skills and knowledge

#### **Role of communication in laboratory**

A laboratory must maintain a healthy relationship between patients, physicians, nursing staff, sales representatives, administrative staff etc. A trained laboratory technologist must know the following aspects of communication

1. Speak to patient clearly with pleasing manners so that his confidence towards the technician increases
2. Communicate correct knowledge to hospital staff on laboratory tests, results and significance of tests.
3. Communication with physicians on interpretation of lab requests and laboratory results
4. Good interpersonal relationship to be observed with co workers.

#### **Organization of a Laboratory**

Only a well-designed laboratory can provide reliable and timely results for the diagnosis of diseases.

The laboratory should be properly planned, so that it will be functional and convenient. The laboratory should have adequate space, ventilation, light, power supply, water supply, work benches, Reagents, Equipment and Laboratory personnel.

An accessible space adequate for setting a desired laboratory is identified. Good ventilated laboratory has reduced risk of infection. Laboratory should have uninterrupted power supply, good lighting system. Good supply of running water and water drainage facility is essential in a laboratory. Work benches should be made at suitable heights with enough leg space. Surface polished Cement benches or wooden benches can be used. Wooden benches are acid proofed. Basic laboratory equipment, glassware and reagents should be made purchased from reputed suppliers. Well trained qualified staff is the back bone of laboratory and the quality of the result depends on the quality of workmanship. A laboratory should have staff pattern including administrator, laboratory technologists, technicians and laboratory assistants for its proper functioning.

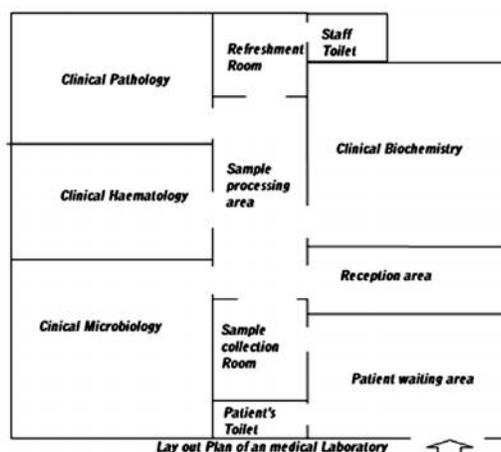
**Components of a Laboratory:** The components of a clinical laboratory include various departments like hematology, blood bank, microbiology, biochemistry, serology and clinical pathology. It also includes reception area, room for specimen collection, refreshment room and toilets

**Lay out plan of a multi- room laboratory:** The working area for different departments as well as sample reception should be conveniently arranged making the necessary reagents and apparatus easily accessible.

#### **Request forms and report forms.**

A request form is a form for lab test signed by the doctor .The request form may carry the patients name, age, IP/OP no., provisional diagnosis, nature of specimen, and name of test required on that specimen. A report form is the form in which the laboratory issues the results of the tests asked for. Various types of report forms for various departments are used routinely. Preprinted and computerised report forms are currently used.

**Maintenance of Stock Registers-** Consumables, Non-consumables .Every laboratory should maintain stock register and each purchase of commodities to the laboratory should be included in it. It is mandatory to maintain separate stock registers for consumables and non-consumables. Reagents chemicals and glass wares are included in consumables. Instruments and machineries used in laboratory and items which will not be exhausted on usage are included in non-consumables.



**Ordering and Utilization of supplies.** A medical laboratory needs an uninterrupted supply of chemicals and reagents for the smooth functioning. Hence prompt ordering and purchasing is important. For this purpose every laboratory personnel should know the details of the item to be purchased and details of various suppliers in that area and merits and demerits of each brand, shelf life period etc.

**Accreditation and Certification of Laboratories.**

Accreditation is used to grade the laboratories having an appropriate quality management system and can properly perform certain test methods and calibration parameters according to their scopes of accreditation. Example for accreditation agencies are NABL, ISO, CAP, CRISIL etc.

**Bar coding**

A major advance in the automation of specimen identification in the clinical laboratory is the incorporation of bar coding technology into analytical systems. A bar coded label (often generated by the laboratory information system and bearing the sample accession number) is placed onto the specimen container and is subsequently "read" by one or more bar code readers placed at key positions in the analytical sequence. The identified information is then transferred to and processed by the system software.

Total Laboratory automation involves the integration and automation of the individual steps of the analytical process. Some manufacturers have developed stand-alone automation system which sort, centrifuge, decap, aliquot, and label tubes. More advanced automation systems provide options such as conveyors to transport specimens, direct sampling interfaces to higher volume analyzers, refrigerated storage and retrieval systems. Now total lab automated system utilizes advanced technologies such as robotics too.

Laboratory information management system (LIMS), often referred to as a laboratory information system (LIS) or laboratory management system (LMS), is a software based laboratory and information management system with features that support whole process of a modern laboratory including operation management, data/records management, quality control, and information sharing among stakeholders including technicians, Physicians & patients etc.

## DETAILING OF PRACTICALS

### **Demonstration of signs and symbols used in laboratory**

Common laboratory signs and symbols collected are distributed for identification and is recorded in the practical log

### **Preparation of different types of laboratory request forms**

Sample request form collected are distributed, prepare a model of it and record in practical log

### **Preparation of different types of laboratory Report forms- Haematology, Biochemistry, Clinincal pathology, Serology, Mixed forms**

Different models of report forms collected are distributed, prepare a model of each and record in practical log

### **Prepare a lay out plan of a multi room laboratory**

A lay out plan is prepared (Chart /model) and is kept as portfolio

### **Preparation of models of stock registers- consumables, Non-consumable**

A model stock register is prepared. Purchase entry and issue of any two items are made and is recorded in the practical log

Stock Register- Non consumable									
Name of item :									Page No:
Sl.no.	Date of purchase	Bill no.& Firm	Quantity	Rate	Amount	Present stock	Balance	Remarks	Initial of Lab in charge
1									
2									
3									

Stock Register- consumable										
Name of item :										Page No:
Sl.no.	Date of purchase	Bill no.& Firm	Quantity	Rate	Amount	Present stock	Issued	Balance	Remarks	Initial of Lab in charge
1										
2										
3										

### **Preparation of model of Order form**

Prepare a model format of order form. It should include name of product , name of manufacturer/ supplier, volume/ pack size, quantity required, specification if any

Demonstration of colour coding for biomedical waste segregation

Chart showing colour coding for biomedical waste segregation are distributed for identification and is recorded in the practical log

### ASSESSMENT ACTIVITIES

- Assignment on Safe Laboratory Practice in Medical laboratory
- Seminar on Common Hazards in a Laboratory
- Chart Preparation on First Aid Practice
- Chart Preparation on different color labels for segregation of biomedical waste
- Model Preparation of Lay out of an ideal laboratory
- Collection of different types of report forms
- Preparation different report forms, & Stock registers
- Preparation of model of a Stock register

### Theory Evaluation Questions

1	Diseases like AIDS, Hepatitis B etc can be transmitted through laboratory specimens if they are not properly handled. Enumerate any three steps adopted to prevent transmission of such diseases to laboratory personnel	3
2	Prepare a model Haematology report form for your PTC Laboratory (Hints: Lab ID, Patient ID, Test Details)	4
3	Given are two important symbols used in a laboratory  <div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;">                       Warning Symbol                 </div> <div style="text-align: center;">                       Prohibitory Symbol                 </div> </div>	4

	a) Differentiate between warning and prohibitory symbols	2
	b) Explain the meaning of the two given symbols	2
4	I am a chemical who is out from the labs now a days. Technician says it due to the fact that I am a carcinogen. Who am I	1
5	Match the following NABL                      Burning of Lab waste HBV infection         Artificial respiration CPR                        Lab Accreditation Incineration            Biological Hazard	4
6	One of your friend hesitate to wear laboratory apron. Advice the friend showing 2 important reasons to use lab apron	2
7	Your lab is having waste disposing bins of different colours. In which bin you will put a used syringe and needle. (Red, Green, Black Blue)	1
8	Prepare and label the lay out for a model laboratory having minimum five sections	5
9	Various personal safety precautions are taken by laboratory personnel during work. Substantiate the statement with examples.	4
10	The different chemicals used in laboratories are hazardous if they are not properly stored or handled with care. Substantiate the statement by giving some proper storing and handling instructions.	4

## Unit 3.2

### CLINICAL PATHOLOGY

A change that takes place in the human body during the process of disease is always reflected in the chemical composition of body fluids. Clinical examination of these fluids reveals the presence of abnormal constituents, altered cellularity, microorganisms and other physical evidences. These evidences from a clinical pathology lab provide endless support to a physician in reaching an early and accurate diagnosis. Apart from the common importance like that of any other laboratory investigation, its importance is paramount in the sense that it includes most of common clinical investigations that are routinely done in a clinical laboratory. Hence an adequate and appropriate understanding of the accurate procedure of these investigations is very essential for a technician. Lack of sufficient automation, decreased sensitivity in microscopy and less specific chemical reactions in the absence of enzyme chemistry are some of the inherent limitations of clinical pathology analysis. Even though the advances in fibro-optic technique enables a pinpoint observation of lower respiratory tract and gastro intestinal tract, the basic analysis of sputum and stool samples still remains in mainstay and so the case of other samples. Easy availability of samples, rapid results, and reasonable precision justifies the need of a clinical pathology lab in a hospital and in the curriculum too. Reporting of positive abnormal finding of the clinical pathology results are important equally to knowledge about the absence of abnormalities for correct diagnosis of a disease.

The analytical tests discussed here are mostly manual types which utilize principles of basic chemical reaction and primarily focuses on physical examination of fluids, microscopy, and simple chemical screening.

#### Learning outcomes

##### The learner:

- identifies different specimens and describes the general guidelines for sample collection in clinical pathology
- discusses the importance of urine analysis
- identifies different type of urine samples and method of collection
- performs physical, chemical and microscopical examination of urine

- performs urine pregnancy test
- identifies the importance sputum analysis
- identifies the importance of stool examination
- discusses the importance of semen analysis and describes the method of semen analysis
- identifies the importance and describes the analysis of C.S.F and other body fluids.
- mentions the recent advances in clinical pathology

### **Introduction**

Clinical pathology is the diagnosis of disease based on the laboratory analysis of body fluids.

Blood, urine, stool, sputum, cerebrospinal fluid, semen, etc are specimens commonly submitted for analysis in a clinical pathology laboratory. Physical, Chemical, Serological and Microscopical examination of specimens are carried out in the laboratory

### **General guidelines for sample collection**

Collect specimens in proper container ie wide mouthed, spill free, disposable containers

Label the specimen such as name of patient, type of specimen, ID no., Date and Time of collection etc.

Follow proper protocol for transportation of specimens

Keep the samples ready for performing analysis.

### **3.2.2 Urine Analysis**

Urine is the most common specimen examined in clinical pathology laboratory. Urine is formed by the kidneys. The body removes water and many harmful waste substances from the body through urine. This is a normal physiological process of the body.

Urine analysis provides mainly diagnostic and prognostic information on conditions like kidney diseases, liver diseases, metabolic disorders, urinary tract infections and parasitic infestations. In addition pregnancy, endocrine disorders and drug overdose are also investigated with a specimen of urine. Simplicity in obtaining the specimen and lesser cost of test makes urine analysis an popular tool in laboratory investigations.

### Types of urine specimen

- Random specimen
- 24 hour specimen.
- Early morning specimen

### Methods of collection

Random specimen is a sample collected without any priority to the time or diet and is collected by the patient in a wide mouth dry container, after cleaning the external genitalia with mild soap and water. Urine specimen from catheter can be obtained in necessary conditions. Urine collection bags are used in case of infants and in geriatrics. A clean, early morning, fasting specimen is generally the most concentrated specimen and is preferred for qualitative, quantitative and microscopic examination for the detection of abnormal constituents.

24 hour urine specimen collection is done by collecting urine in a special container, 2.5 to 5 litre capacity with added preservative. Preferably morning 8 am to next day 8 am over a period of 24 hr. It helps to diagnose kidney problems. It is often collected to do tests like clearance tests and to measure protein, hormones, minerals, and other chemical compounds. The preservatives are added to prevent changes in the urine (pH, odour, bacterial growth etc) that may happen on standing longer periods. Preservatives commonly used are toluene, formaldehyde, chloroform, boric acid, hydrochloric acid etc.

### Physical examination of urine

All routine urine analysis should begin with a physical examination of the urine sample. This examination includes assessment of appearance (colour and transparency), specific gravity, volume, reaction and odour. For best results of the physical examination of urine the specimen should be evaluated immediately after collection.

#### Colour

Normally the urine is straw coloured (due to urochrome). Colour depends on the concentration of urine and varies with the presence of constituents. Normal colour of urine changes in different disease conditions or in persons taking medications.

Deep Yellow : Mild to severe dehydration, Jaundice, B complex therapy

Red to brown : Haematuria, haemoglobinuria, myoglobinuria, porphyria

Brown to black : Alkaptonuria, methaemoglobinuria.

Milky : due to the presence of lymph (Chyluria)

The colour of urine is usually described after visual inspection with common colour terms.

### **Transparency**

Freshly voided urine is clear and transparent. It may become turbid if exposed for long time due to the urea being acted upon by bacteria or the presence of proteins, pus cells, blood, bacteria, urates, phosphates.

### **Specific gravity**

It implies the capacity of kidney to concentrate urine. Normal specific gravity of urine is 1.010-1.025

Values more than 1.025 indicate severe dehydration, DM (diabetes mellitus), Adrenal insufficiency. Values less than 1.010 indicates increased water output as in Diabetes insipidus. A low fixed specific gravity usually found is chronic renal failure. It is measured by urinometer or refractometer.

### **Volume**

Normal urinary output ranges between 1000 - 1500 ml / day. It depends upon fluid intake, solute load, loss of fluid by skin or otherwise, climatic condition etc

A urine out put more than 3 litre/day is called Polyuria and is seen in- Diabetes Mellitus, Diabetes Insipidus, Recovery from acute renal failure (ARF), Diuretic therapy etc.

A urine output Less than 500 ml/day is Oliguria and is seen in Acute renal failure, Vomiting, Fever, Burns.

Anuria is the suppression of urine output (generally less than 50 ml per 12 hours is considered as anuria)

Urine volumes can be measured usually with a volumetric cylinder

### **Reaction (pH)**

Normal fresh urine is acidic approximately 6. Depending on the person's acid-base status, the pH of urine may range from 4.5 to 8.

A Urine pH 8.5 or more may be found after heavy meals, Urinary tract obstruction, Chronic renal failure

A pH 4.5 or less may be seen after heavy exercise, Metabolic Acidosis, Chronic Respiratory, Acidosis, Uncontrolled diabetes

Extremely acidic or alkaline urine usually indicates a poorly collected specimen.

Litmus Paper, Reagent strip testing(pH paper), are commonly employed for measurement of pH,

### **Odour**

Freshly voided urine is slightly aromatic. Urine becomes more ammoniacal due to bacterial activity. A fruity odour is felt in case of severe diabetes due to presence of ketone bodies. A putrid or foul smell is felt in Urinary tract infections.

### **Chemical Examination of Urine**

Chemical examination of urine is done to detect presence or absence of certain constituents which reflects disease state of body. The parameters include Protein, Sugar, Ketone bodies, Blood, Bile pigments, Bile salts and Urobilinogen. Conventional test methods are commonly employed. Reagent test strips (dip sticks) are commercially available and recently used.

#### **1. Proteins**

Normally very small amount of protein (100 mg/day) is excreted and is not detectable by routine qualitative test methods. Proteinuria is the term used for increased protein excretion in urine. Commonly found protein in urine is albumin so it is often termed as albuminuria. Proteinuria is seen in kidney diseases such as Nephrotic Syndrome, Multiple myeloma, chemical poisons etc.

#### **Methods of detection**

The methods are based on the principle of precipitation of protein by chemical agents or coagulation by heat

- Heat and Acetic acid method
- Sulphosalicylic acid method
- Reagent test strips (dip sticks) like albustix

#### **2. Sugar**

Glucose is the predominant sugar and is normally absent in urine. The renal threshold for glucose is 180 mg%. If the blood sugar rises above renal threshold level, the glucose will appear in the urine and is called glycosuria. Major cause of Glycosuria is Diabetes mellitus. Transient glycosuria is seen in pregnancy, stress etc.

#### **Methods of detection**

- Benedicts test--When Benedicts qualitative reagent is heated with urine, glucose present in urine reduces cupric ions present in the reagent to cuprous ions. Alkaline medium is provided to the reaction by sodium

carbonate present in reagent. The color changes to green, yellow, orange or red according to the concentration of glucose in urine.

- Reagent test strips (dip sticks) like glucostix.

### 3. Ketone Bodies

Ketone bodies is a term used to describe three discrete but metabolically related chemicals-Acetone, Acetoacetic acid and  $\beta$  - hydroxy butyric acid. Ketone bodies are spilled into urine and the presence of Ketone body in urine is called ketonuria. Ketone bodies are detected in conditions such as diabetic ketoacidosis and starvation.

#### Methods of detection

- Rothera's test ( Ketone bodies form purple coloured complexes with sodium nitroprusside in alkaline medium)
- Reagent test strips (dip sticks) like ketostix

A fresh urine specimen is always preferred for ketone body estimation.

### 4. Blood

Presence of blood in urine is called Haematuria, seen in cases of Nephrolithiasis (urinary calculi) and in malignancy. A microscopic examination of urine helps to confirm the presence of intact erythrocytes.

Healthy individuals normally will have no detectable blood in their urine.

#### Methods of detection

- Benzidine test - It is the test for occult blood. It is highly sensitive but as benzidine is considered as a potent carcinogen, this test is no longer used now.
- Reagent test strips ( Haem occult test)

### 6. Bile Pigments

These are breakdown products of haemoglobin excreted in bile. The two most important bile pigments are bilirubin, which is orange or yellow, and its oxidized form biliverdin, which is green.

Normally bile pigments are absent in urine. Presence of bile pigments in the urine indicates liver dysfunction such as obstructive jaundice. Fresh urine sample should be used for bilirubin determination because exposure of urine to light may cause loss of bile pigment by oxidation and leads to negative result.

**Methods of detection**

- Fouchet's test. (Bilirubin is treated with ferric chloride in Tri chloro acetic acid to give greenish blue colour)
- Reagent test strips (dip sticks)

**7. Bile Salts**

Bile salts are made in the liver from cholesterol and these help in the fats absorption. Sodium taurocholate and sodium glycocholate are the major bile salts. Normally bile salts are negative in the urine. The presence of bile salt in the urine indicates disease like Obstructive Jaundice.

**Methods of detection**

- Hay's test. (Bile salts when present, lower the surface tension of urine. When sulphur powder is added to the urine, sulphur particles sink to the bottom of the tube indicates positive result.)

**8. Urobilinogen**

Majority of Bile pigment derived from breakdown of hemoglobin is excreted in the stool, but small amounts are reabsorbed into the blood from the intestines and then excreted into the urine. Urobilinogen is formed in the intestines by bacterial action on bilirubin. Urobilinogen is normally present in urine in low concentrations. A fresh specimen is essential for the detection of urobilinogen, as it is a light-sensitive compound. Positive test results help to detect liver diseases such as hepatitis, cirrhosis and haemolytic anaemia

**Methods of detection**

- Ehrlich's Test (Urobilinogen forms a cherry red complex with para dimethyl amino benzaldehyde in normal urine. On further dilution of sample (>1:20) a positive result is significant.)

**Microscopic examination of Urine**

Microscopic Examination of urine sediments provides a direct sampling of urinary tract morphology, which helps the diagnosis very much.

The specimen used for microscopic examination should be as fresh as possible. Red cells and many formed solids tend to disintegrate upon standing, particularly if the specimen is warm or alkaline. Concentrated first morning, mid-stream, clean catch urine specimen, is preferred for microscopy. Urine is collected without faecal & vaginal contamination.

The urine sediments assessed under microscope can be of two types

- **Organized** - Leukocyte (Pus cells), Erythrocytes, Casts, Epithelial cells, Bacteria, Parasites and fungi.
- **Unorganized** - Crystals and amorphous sediments.

**Pus cells** - They are round or oval in shape and present normally, 0 to 3 leukocytes per high-power field will be seen on microscopic examination. The pus cells are leucocytes. More than 5 cells per high-power field probably indicate urinary tract infection.

**Erythrocytes** - Red cells are not usually present in normal urine and appear as refractile bodies, seen in acute glomerulo nephritis, stones in urinary tract and malignancy etc. Estimate their number per high-power field and report it.

**Epithelial cells** - Normal urine shows a few epithelial cells. Marked increase in the number of epithelial cells signifies some pathological conditions of the site of their origin.

**Bacteria** - Presence of numerous bacteria in the centrifuged deposit of freshly voided urine signifies infection in the urinary tract.

**Casts** - These are formed by coagulation of albuminous material in the kidney tubules. Casts are cylindrical and vary in diameter. The sides are parallel, and the ends are usually rounded. Casts in the urine always indicate some form of kidney disorder and should always be reported

There are different types of casts. They are Hyaline casts, Red cell casts, Granular casts, Epithelial casts, Waxy casts and Fatty casts

### **Crystals**

Crystals of various substances can be seen according to the reactions (pH) of urine.

#### **Crystals found in normal acid urine-**

- Amorphous urates- yellow and granular precipitate
- Uric Acid Crystals appear in several forms, Multi colored when polarized most commonly Diamond shaped
- Calcium Oxalate Crystals is most frequently observed in urine and octahedryl in shapes, often referred to as an 'envelope' shape

#### **Crystals found in Alkaline urine**

- Amorphous phosphates-fine granular precipitates
- Calcium phosphate- stilette prism

- Calcium carbonates- colourless spheres or dumbbell shaped
- Triple Phosphate Crystals are Colorless, 4-6 sided prisms Referred to as 'coffin lid crystals'

**Crystal found in abnormal urine**

Cystine - colourless, retractile, hexagonal

Tyrosine - Fine needle in clumps, yellow and silky

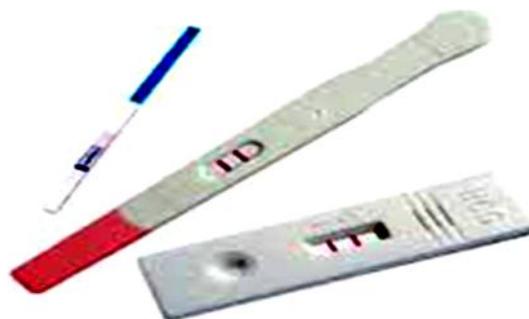
Leucine - yellow oily spheres

Sulphonamide - yellow brown striated sheaves or round with radial striations



**Urine Pregnancy Test**

Most chemical tests for pregnancy look for the presence of the beta subunit of hCG, or human chorionic gonadotropin, in the blood or urine. hCG can be detected in urine or blood after implantation, which occur six to twelve days after fertilization.



The common test done for detection of hCG in Urine is Card Test. It qualitatively detects the presence of hCG. It is a single step immunoassay and has high sensitivity. Paper strips coated with monoclonal anti-hCG antibody incorporated in a disposable card is used for the test. The urine is introduced in the sample window. Appearance of coloured bands in the control and test areas denote a positive test.

### Urine Analyser

Automated machines are now available in the clinical laboratory to perform urine analysis called 'Urine Analyser'. Using urine strip readers, the unit can detect and quantify a number of analytes including bilirubin, protein, glucose, red blood cells etc. The instrument works on the principle of Reflectance photometry and can process several hundred strips per hour. Different types of urine analysers include Cobas 6500, UF-1000-SIEMENS CLINITEK ANALYSER etc.



## DETAILING OF PRACTICALS

### Detection of Protein in Urine - Heat and Acetic Acid Method

1. Cloudy urine specimens are filtered or centrifuged
2. Fill clear urine 2/3 of a small test tube
3. Boil the upper portion of urine over a flame by keeping the tube in a slanting position. (lower half serves as control)
4. If turbidity develops, add 1-2 drops of 3-5% acetic acid solution. Sometimes turbidity may be due to phosphate or carbonate precipitation. If it is so then glacial acetic acid clear up the turbidity. If it is due to protein then precipitation will be there after the addition of acetic acid.
5. Reboil the specimen
6. If turbidity is present protein is present .if there is no turbidity in upper portion then protein is absent.
7. Grade the turbidity as follows:

- Negative : No cloudiness
- Trace: Barely visible/ slight cloudiness.
- 1+ : definite cloud without granular flocculation
- 2+ : heavy and granular cloud without granular flocculation
- 3+ : dense cloud with marked flocculation.
- 4+ : thick curdy precipitation and coagulation

### Detection of sugar in Urine - Benedicts Test

#### Procedure

1. Pipette 5ml of benedicts reagent in test tube
2. Add 8 drops of urine
3. Heat carefully over a flame for 1-2 minutes or place in boiling water bath for 5 minutes.
4. Cool under tap water.
5. Note the colour of solution and precipitate.

Interpret as follows

No change in color i.e. blue	: Absence of sugar.
Green	: Trace
green with yellow precipitate	: 0.5%
Yellow	: 1%
Orange	: 1.5%
Brick red	: $\geq 2\%$

### Detection of Ketone bodies in Urine - Rothera's Test

#### Procedure

1. Transfer about 5 ml of urine to a test tube
2. Add solid ammonium sulphate a little at a time with mixing to saturate the urine.
3. Add a pinch of sodium nitroprusside or 2-3 ml of solution
4. Mix well and add liquor Ammonia drop wise along the sides of the tube
5. Observe purple ring at the junction of two layers

Appearance of purple ring indicates the presence of ketone bodies

### **Detection of Bile pigment in Urine - Fouchet's Test**

#### **Procedure**

1. Take 5 ml of urine in test tube
2. Add 2 ml of barium chloride and mix well
3. Filter through filter paper
4. Unfold the filter paper and spread it on the dry filter paper.
5. Add 1- 2 drops of Fouchet's reagent on the precipitate
6. A green or blue color indicates presence of bilirubin.

### **Detection of Bile salt in Urine - Hays test**

#### **Procedure**

1. Take 2ml of urine in a test tube or small beaker
2. Sprinkle little amount of fine sulphur powder
3. Observe the movement of sulphur powder without shaking the beaker
4. Sinking of sulphur powder indicates presence of Bile salts

### **Microscopic examination of urine**

1. Transfer urine sample to a conical centrifuge tube.
2. Centrifuge your sample at a moderate speed 1500 - 2500 rpm for 5 minutes.
3. Discard the supernatant by quickly pouring off fluid.
4. Tap tube with index finger to mix sediment with remaining fluid.
5. Make a wet mount of sample by transferring 1 drop of material to a slide and covering with a coverslip.
6. Examine the sample under the microscope under low and high power.
7. Identify the sediments observed and report their approximate number in Lpf/Hpf.

### **hCG detection in urine- Urine Pregnancy card test**

1. Open the sealed pouch of the test card and bring the card to room temperature
2. Add three drops of urine through sample window using dropper provided. Wait for coloured bands to appear.
3. Appearance of coloured bands in the control and test areas denote a positive test

### 3.2.3 Sputum examination

Sputum is mucus that is coughed up from the lower airways of the respiratory tract i.e lungs, trachea and bronchi. Saliva and nasopharyngeal secretions are not part of the sputum. Sputum samples are used for microbiological investigations of respiratory tract infections and cytological investigations of cancer. Purulent sputum contains pus, composed of white blood cells, cellular debris, dead tissue, serous fluid, and viscous liquid (mucus).

#### **Collection:**

An early morning sample obtained by deep cough is a preferred sample for analysis, For the diagnosis of Tuberculosis, sample may be collected on three consecutive days. Wide mouthed disposable containers are preferred for the collection of sputum sample. The patient should take a deep breath, and empty his lungs in one breath and at the same time cough as hard and deeply as he can. Whatever he brings up by coughing, should be spit into the container and collected.

#### **Physical Examination**

It involves measurement of colour and consistency or appearance. Normal sputum is pale yellow in colour and contains mucoid materials. It may be red in Pulmonary Tuberculosis due to the presence of blood (Haemoptysis), dark yellow in bronchitis and brown in pneumonia.

Normal sputum is viscous due to the presence of mucus, purulent in cases of bronchitis or acute upper respiratory tract infection, frothy in pulmonary oedema.

#### **Microscopic examination**

It involves mainly the examination of unstained and stained smears. For preparing sputum smears for microscopic examination, the purulent portion of the sample must be included. While handling utmost care should be taken in suspected cases of TB. Examination of Ziehl Neelson stained smears are most routinely used method in the diagnosis of Pulmonary Tuberculosis.

Grams stained smears are commonly used for the detection of bacteria causing respiratory infections such as Pneumococci, Haemophilus and staphylococcus. Stained and unstained smears are used to detect the presence of puscells, epithelial cells, RBCs, malignant cells, Curshmann spirals, Charcot leydon crystals etc.

## DETAILING OF PRACTICALS

### Demonstration of Ziehl Neelsen Staining Technique

Prepare a smear from the purulent portion part of the sputum using an applicator stick, heat fix and do ziehl nelson staining, air dry and look under oil immersion field of microscope for the presence of red coloured rod shaped organism in a blue back ground, number of organisms noted and graded as 1+ to 4+ in the report. Presence of tubercle bacilli indicates tuberculosis infection.

### 3.2.4 Stool Analysis

Stool is an important specimen for the diagnosis of diseases of gastrointestinal tract such as diarrhoea, dysentery, parasitic infection, gastrointestinal bleeding, peptic ulcer, carcinoma and malabsorption syndromes. Tests which reveal the presence of blood denotes an ulcerative lesion somewhere in the gastro intestinal tract, either inflammatory or as a result of cancer. Examination of stool shows the presence of parasites such as Entamoeba histolytica, various types of tape worms, pin worms, round worm and its egg.

Collection of Specimen: A portion of fresh specimen is collected in a clean wide mouthed container with a spatula. Areas containing mucus, blood and pus should be incorporated in the specimen.

Analysis includes Physical, Chemical and microscopic examinations

#### Physical examination-

Macroscopic examination of the stool involves consistency, colour, blood, odour, pH etc. The presence of adult worms in a freshly passed stool (adult stages of Ascaris lumbricoides and Enterobius vermicularis, Proglottids of Taenia species) is also reported.

#### Chemical examination

It includes the tests for occult blood and reducing substances

Blood may be present hidden in the stool called occult blood and is seen in malignancies, ulcers, haemorrhoids etc. Occult blood in stool were detected by Benzidine based tests, and is now not used due to the carcinogenicity of benzidine. It has been replaced by strip tests.

The presence of reducing substances is usually done in children suspecting lactose intolerance. The unabsorbed sugars in stool are measured as reducing substances. Benedict's test is commonly employed for detecting reducing substances.

### **Microscopic examination**

Microscopic examination of faeces can be done by saline preparation and iodine preparation. It helps detection of identification of cells, crystals, protozoa, ova of intestinal parasites

#### **Direct saline wet mount**

- a. Place a drop of saline on the slide.
- b. Pick up a small amount of faecal material on the end of an applicator stick
- c. Emulsify in the saline and cover with a cover slip. Examine on low and high power. The smear should be thin enough to read a printed page through it.
- d. The entire preparation must be examined for the presence of eggs, larvae and protozoa.

#### **Iodine wet mount**

Iodine preparation is useful for the identification and differentiation of protozoal eggs and cysts

- a. Place a drop of Lugol's iodine solution on a slide.
- b. Pick up a small amount of fecal material on an applicator stick
- c. Emulsify in the iodine solution and cover with a coverslip.
- d. Examine on low and high power as described in the previous procedure.

### **3.2.5 Semen Analysis**

Semen is a composite secretion formed by testes (male reproductive organ) as well as the accessory glands. Semen analysis is also called 'Seminogram' evaluates certain characteristics of semen and the sperm contained therein. It is done to evaluate male fertility or verifying the success of vasectomy or suspected cases of paternity in medico legal disputes. As a result of its relative simplicity, semen analysis is first requested before more complicated and expensive examination of the female in the infertility treatment.

#### **Specimen collection**

The sample should be collected after a minimum of 3-5 days of sexual abstinence. The sample should be obtained by masturbation and ejaculated into a clean, wide-mouthed container made of glass or plastic. It should be emphasized that the semen

sample needs to be completely collected and reached in the laboratory within 30 minutes of collection, in order to limit the exposure of the semen to fluctuations in temperature.

Semen analysis should begin with a physical (gross) examination soon after liquefaction, preferably at 30 minutes, but no longer than 1 hour after ejaculation.

Physical examination includes volume, viscosity, colour, reaction and liquefaction time

**Liquefaction time:**

Semen is typically a semisolid coagulated mass. Within a few minutes at room temperature, the semen usually begins to liquefy and complete within 30 minutes. The time taken to liquefy the sample is noted.

**Macroscopic examination of semen**

**Semen viscosity:**

The viscosity of the sample can be estimated by gently aspirating it into a plastic disposable pipette, allowing the semen to drop by gravity and observing the length of any thread. A normal sample leaves the pipette in small discrete drops. If viscosity is abnormal, the drop will form a thread more than 2 cm long.

**Colour:**

A normal liquefied semen sample has a grey-opalescent appearance.

It may appear less opaque if the sperm concentration is very low.

The colour may be red-brown when red blood cells are present (haemospermia), yellow in jaundice or in taking certain vitamins or drugs.

Volume:

Normal volume of semen is between 1.5-5 ml. Volume of sample is usually measured by collecting the sample directly into a modified graduated glass measuring cylinder.

Reaction (pH): Reaction of semen is normally alkaline (pH 7.2-8.9), decrease in pH interfere with semen motility.

**Microscopic examination:**

During the microscopic examination of the sample, estimation of motility, sperm count and evaluation of sperm morphology is performed. The presence of cells other than spermatozoa and clumping of spermatozoa is also to be observed.

**Detection of Motility:**

A drop of well mixed liquefied semen is placed on a clean glass slide and put a cover glass. Then examine under high power objective for the motility of spermatozoa in at least 10 fields report in percentage and graded as

- Rapid progressive motile (Active).
- Slow progressive motile (sluggish)
- Immotile

**Sperm Count**

The number of sperms in sample can be estimated in a counting chamber after diluting the semen. After liquefaction, gently mix the specimen and draw it up to the 0.5 mark of a WBC pipette. Then draw the diluting fluid (Sodium bicarbonate-Formalin fluid) up to the 11 mark and mix well. Charge the counting chamber, keep for 5 minutes and then count the number of sperms in the four corner squares under low power objective. Count only those spermatozoa which are complete i.e. with head body and tail. Calculate the number of spermatozoa/ml of the sample by multiplying the number of sperms counted by 50,000. The normal Sperm count is 80-160 millions /ml.

Oligozoospermia refers to seminal fluid in which contain decreased number of spermatozoa.

Azoospermia is a condition in which semen contains no sperm.

Necrozoospermia refers to semen in which all sperm are non-viable or non-motile.

**Sperm morphology**

Sperm morphology is evaluated by examining the stained smear. After assessing approximately 200 spermatozoa, report the percentage of normal and abnormal forms. Normal semen contains less than 20% abnormal forms.

**Normal sperm morphology**

Spermatozoa consists of a head, neck, middle piece (midpiece), endpiece. For a spermatozoon to be considered normal, both its head and tail must be normal. All borderline forms should be considered abnormal.

- \_ The head should be smooth and oval in shape
- \_ The midpiece should be slender, regular and about the same length as the sperm head.

- The end piece should be thinner than the mid piece, and longer, about 10 times the head length.

### **Chemical examination**

Chemical analysis of semen sample involves the detection of the following analytes:

- Acid Phosphatase - To evaluate the secretory function of the prostate and used in forensic analysis
- Fructose - since fructose is the source of energy to sperm, fructose concentration indicates viability of sperms in sample.

### **3.2.6 CSF And Other Body Fluids**

#### **CSF Examination**

CSF (cerebrospinal fluid) is a clear watery fluid circulating in the sub arachnoid space around the spinal cord and brain. It protects the brain and spinal cord from injuries, also acts as a transport medium for transport of substances between the tissues of brain spinal cord and blood. Examination of CSF is done for the diagnosis of various diseases like meningitis, tumours of central nervous system

CSF is collected by lumbar puncture under aseptic precautions. A special type needle (Lumbar puncture needle) is used for specimen collection. Usually collected by an experienced clinician. The puncture site is cleaned with iodine and spirit. The needle is carefully introduced into the subarachnoid space. When the needle tip is reached the correct depth remove the stylet. CSF will flow through the needle. In adults the needle is usually administered between the spines of 3rd and 4th lumbar vertebra. In small children it is done between 4th and 5th lumbar vertebra. CSF pressure is also measured using a glass manometer attached to the needle. 6 to 8 ml CSF is usually collected for routine examination in three sterile containers. First few drops in the first container for bacteriological investigations, major portion in the second tube for bio chemical investigation and the rest for cytological investigation in the third tube. Examination of CSF should be done within 1 hour after collection.

#### **Physical Examination**

Normal CSF is clear having the specific gravity 1.003. Turbidity may be due to the presence of RBC leucocytes or bacteria, Yellow colour due to old haemorrhage, severe jaundice or spinal constriction. Bloody due to trauma during collection sub arachnoid haemorrhage. In tuberculosis meningitis the clot appears as a cob web.

### **Microscopic Examination**

Done to evaluate the cellular constituents of CSF. Should be done soon after the collection. Normal CSF contains lymphocytes only. Polymorphs are present only in pathological conditions. normal cell count is 0-5 lymphocytes per cumm.

Cell count is done by diluting 1 in 10 with diluting fluid and counting is done using Improved Neubaur counting chamber or Fuchs Rosenthal counting chamber. The number of cells per ml of undiluted CSF is counted and calculated. Increased cell count is seen in meningitis, encephalitis, poliomyelitis, syphilis etc.

### **Differential Cell Count**

Sample having an increased cell count is subjected to differential count. Prepare a smear using centrifuged sample dry and stain with Lieshman's stain, examine under microscope. Increased lymphocyte percentage indicates viral infection and increased neutophils indicates bacterial infection.

### **Chemical Examination**

Chemical Examination includes glucose, protein and chloride. Normal protein content is 15-40mg%. Globulin is the major protein present. CSF protein is increased in acute meningitis, sub arachnoid haemorrhage etc. Sulphosalicylic acid test is usually used for CSF protein determination.

Normal CSF glucose level is 40-70mg%, level is usually decreased in bacterial meningitis, small increase is noticed in poliomyelitis, encephalitis brain tumours etc. In diabetes the CSF glucose level is increased normally about 60-80% of the blood glucose level. Methods employed for blood glucose estimation can be used for CSF Glucose estimation also.

Normal CSF Chloride level is 115-125 mEq/l and it is decreased in all types of meningitis, CSF Chloride is usually estimated by titrimetric method.

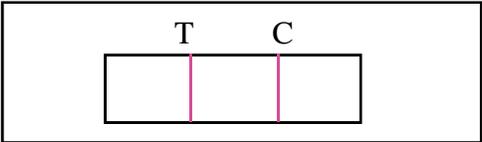
Other body fluids include Pleural, pericardial, peritoneal fluid, Ascitic fluid, synovial fluid, gastric juice etc.

The general approach of laboratory study is the same as the CSF-physical examination, microscopic examination, bacterial culture and chemical examination. Synovial fluid is the fluid found around the joint examined in order to assist in the diagnosis of joint-arthritis, gout or infection of the joint (septic arthritis).

### ASSESSMENT ACTIVITIES

- Performance of urine analysis with the given sample and record the results
- Assignment given on importance of sputum examination in Tuberculosis
- Assignment given on how Microscopic examination of stool is performed
- Flow chart Preparation showing different procedures in semen analysis
- Chart Preparation showing precautions taken during collection of semen

### Theory Evaluation Questions

1	Your MLT Teacher asks your group to present a seminar on Semen Analysis a) Prepare sub topics for the members b) Explain the procedure for determining the Total sperm count c) With the help of a labeled diagram explain the structure of a normal spermatozoa	3 5 3
2	CSF collection a complex process. Name the method used for this	1
3	The given diagram shows the result obtained while doing urine pregnancy test for a patient  a) Interpret the result (1 score) b) Write the principle and procedure of the test( 3 score)	1 3
4	Give reasons for the following A. Just a positive test for urine urobilinogen doesn't have much clinical significance B. Sulphur powder sinks to the bottom of urine sample positive for bile salts	1 1
5	Prepare an instruction notice to patients regarding sputum sample collection.	3
6	Find "me" using the clues i) I am an instrument used to check specific gravity. Temperature correction is not needed ii) I am a hormone. I am normally found in the urine of pregnant woman	2

## Unit 3.3

### CLINICAL BIOCHEMISTRY

Clinical Biochemistry is one of the most rapidly advancing areas of a clinical laboratory which deals with various biochemical parameters of the body. The marked increase in the number and availability of biochemical determinations has evolved the advancement of laboratory medicine to a highly sophisticated molecular level. Advances in technique, practicing standards, and interpretation in this field have made the area, most multifaceted and complex. This unit of Clinical Biochemistry will make the learner familiar with the basic biochemical analytical procedures as well as to get aware of the recent trends in clinical biochemistry.

This unit aims at the importance of emphasizing the application of clinical biochemistry to medicine. This unit gives the basic theoretical and practical information's in clinical biochemistry which are used for the diagnosis and treatment of diseases. In a clinical laboratory most of the investigations which the physicians rely on are from clinical biochemistry. Every disease has a biochemical origin, which may alter the biochemical parameters. These parameters are estimated from the body fluids by processing different specimens. Learners should know the basic requirements for the biochemical investigations including different biological specimens, their collection and processing of biochemical estimations and have brief knowledge of preparation of solutions and different types of assays. The learner should familiarize common instruments and the working procedures. The module suggests students activities a step by step guide to perform few of the biochemical estimation procedures to practice the procedures taught in the lessons. The module covers the routine biochemical investigations like blood sugar, renal function tests, Liver function tests, lipid profile and relevance of other clinical biochemistry estimations.

#### Learning outcomes

##### The learner:

- defines clinical biochemistry, grades of various chemicals and preparation of solutions
- differentiates different types of assays used in biochemistry.
- identifies different types of specimens, their collection and processing for biochemical analysis.

- performs cleaning of glass wares
- explains the parts, working, use and to operate common instruments used in biochemistry
- explains Diabetes and differentiates various blood samples used for blood sugar estimation
- identifies different blood sugar estimation methods and estimates blood glucose by GOD POD method
- explains GTT, GCT procedures, Glucometer technique and importance of HBA1c
- explains the relevance of renal function test and to identify various tests included in the RFT panel
- identifies common blood urea estimation methods and estimate blood Urea by Berthlot method
- identifies common creatinine estimation methods and to estimate S.Creatinine by Jaffes method
- discusses the importance of uric acid and estimation of uric acid by uricase method
- identifies the importance of microalbumin, Cystatin-C and clearance tests for the evaluation of renal function
- explains the relevance of Liver function test and to identify various tests included in the LFT panel
- explains jaundice and to perform Estimation of Bilirubin by Malloy- Evelyn method
- performs the estimation of serum total protein by Biuret method
- performs the estimation of serum albumin by BCGmethod
- identifies the importance of ALT,AST&ALP parameters in the evaluation of Liver function
- explains the importance of Lipid profile and to identify various tests included in the Lipid profile
- performs the estimation of S. Cholesterol by CHOD-PAP method
- identifies the importance of S.Electrolytes
- identifies the clinical significance of S.Calcium & Phosphorous estimations
- identifies the clinical significance of various special biochemical tests

- identifies the importance of quality control in biochemistry and able to explain various terms used in quality control
- identifies the need, advantages and recent advances of automation in a Clinical biochemistry laboratory
- mentions the advanced diagnostic methods in clinical biochemistry

### 3.3.1 Introduction to biochemistry

The human body is a complex structure and many chemical reactions take place in the human body, Biochemistry deal with the understanding of various biochemical reactions occurring in an organism. Clinical biochemistry involves analytical measurement of different chemical constituents of the body fluids like serum, plasma, urine, CSF etc at the molecular level. The results that are obtained from such measurements are compared with reference ranges and reflects the malfunctions of different organs like liver, kidney, pancreas, etc. there by helping in the diagnosis of diseases and treatment.

#### Types of Chemicals

Based on purity, different grades of chemicals are used in the laboratory; Routine laboratory uses Laboratory grade (LR/GR) reagents. Analytical grade reagents (AR/Analar) are chemicals of high purity used for advanced laboratory procedures.

Chemicals can also be classified into primary standards which are available in pure crystalline form and do not absorb water, so they retain their form and structure and stored for a considerable period of time eg. Oxalic acid, sodium carbonate etc. others such as sodium hydroxide, hydrochloric acid etc. are grouped as secondary standard chemicals .

#### Solutions-

Solution is a homogenous mixture of solvent and its dissolved solute. There are different types of solutions used in medical laboratory procedures which includes

- a) Percentage solutions

This is the most commonly used solution type in a medical laboratory. Percentage solution contains grams of solute per 100 ml of solution. Percentage solution may be W/V or V/V solutions. In weight per unit volume (W/V) solution, particular weight of solute is dissolved in a liquid solvent and volume per unit volume (V/V) solution is prepared from liquids.

## b) Saturated solution

A saturated solution is the solution that contain maximum amount of dissolved solute.

## c) Molar Solution

A solution that contains gram molecular weight of solute dissolved in one litre of solution. For example, the molar weight of sulphuric acid ( $\text{H}_2\text{SO}_4$ ) is 98. Therefore, one mole of  $\text{H}_2\text{SO}_4$  contains 98 gm of  $\text{H}_2\text{SO}_4$  per liter of solution.

## d) Normal Solution

A solution that contains gram equivalents of a solute dissolved in one litre of the solution. The equivalent weight of  $\text{H}_2\text{SO}_4$  is 98 divided by 2 (valancy of  $\text{H}_2\text{SO}_4$ ), which is 49. Therefore, one normal solution of  $\text{H}_2\text{SO}_4$  contains 49 gram of  $\text{H}_2\text{SO}_4$  per litre of solution.

## e) Standard solution

Standard solution is a solution whose concentration is exactly known.

**Assay**

Assay is the term referred to the analytic procedures in a laboratory, for qualitatively assessing or quantitatively measuring the presence or amount or the functional activity of an analyte.

In an assay, when an analyte is detected using a chemical reaction, there are two options for assessing its concentration. One is to wait until the reaction is complete and the total amount of analyte is converted to product - called an endpoint reaction. The other is to measure the rate of change in product formed over time - called Kinetic or rate reaction.

**Endpoint Assay**

Endpoint reactions are especially suitable for chemical reactions which are completed in a relatively short time and they produce one product for each molecule of analyte. For example, a reaction of albumin with the dye bromocresol green (BCG) produces a colored complex. If the reaction is allowed to continue until all the albumin present in solution has reacted and the maximum amount of colored product has formed, the colour at the end of the reaction reflects the total amount of albumin as the albumin-dye complex.

**Kinetic Assay**

Enzyme activity is mostly determined by a rate reaction rather than an endpoint reaction. In such cases determination of the enzyme concentration is based on how

fast a fixed amount of substrate is converted to product. The more enzyme present, the faster the conversion. Examples of enzymes that are often measured in the clinical laboratory include SGPT (an enzyme measured in Liver diseases)

Kinetic assay may also be used for measurement of analytes that are not enzymes. For example, if a reaction is very slow to reach an endpoint, a rate method may be more practical in order to obtain a result in a shorter time frame. Some examples of analytes other than enzymes that are measured using rate reaction include ammonia (a waste product of protein metabolism).

### **Common Specimens used in clinical biochemistry**

Body fluids frequently used for testing include blood, urine, saliva, cerebrospinal fluid (CSF), amniotic fluid, synovial fluid, pleural fluid, peritoneal fluid and pericardial fluid etc. These fluids often contain the same analytes of interest - such as glucose and protein - but differ greatly from each other in physical and chemical properties.

Blood is the most commonly used specimen for testing in the clinical laboratory. Serum or plasma part of the blood is commonly used for biochemical investigations.

Urine is another fluid commonly used for testing in clinical chemistry laboratories. It is especially suitable for tests that evaluate kidney functions, tests that look at waste products that are excreted by the kidneys, and for metabolites like hCG etc. Sometimes both serum and urine concentrations of a substance are useful to know in order to evaluate how well the analyte is being excreted.

Fluids other than blood and urine, like amniotic fluid, synovial fluid, peritoneal fluid, pleural fluid and pericardial fluid, are used in limited clinical settings and are tested for only a few special analytes.

### **Cleaning of Glassware for biochemical analysis**

Good laboratory technique demands clean glassware. Tests may lead to erroneous result if dirty glassware is used.

The used glass wares should be washed with water immediately after use, soak in mild detergent solution, wash in running tap water and rinse with distilled water and dry. If lab-ware is not cleaned immediately, it may become impossible to remove the residues. Such glass wares should be soaked in water or special cleaning solutions. Special cleaning solutions such as chromic acid solutions are suggested in the case of more dirty glass wares.

Most new glass wares are slightly alkaline in reaction. New glassware should also be soaked in several hours in acid water (a 1% solution of hydrochloric or nitric acid) before use.

Pipettes should be placed in a vertical position with the tips up in a jar of cleaning solution. A pad of glass wool is placed at the bottom of the jar to prevent breakage. After soaking for several hours, the tips are drained and washed under running tap water until all traces of cleaning solution are removed. The pipettes are then rinsed in distilled water and are dried in an oven at not more than 110°C.

Most laboratories that use large numbers of pipettes daily, use a convenient automatic pipette washer. These devices are made of metal or polyethylene and can be connected directly to hot and cold water supplies. Polyethylene baskets and jars may be used for soaking and rinsing pipettes in chromic acid cleaning solution.

It is important that the necks of volumetric flasks above the graduation mark be clean because, when solutions are diluted in the flask, drops of water may adhere to an unclean wall and may invalidate the measurement of volume.

### 3.3.2 Instruments used in Biochemistry

Clinical biochemistry involves different analytical methods for the measurement of various biological compounds in the body fluids. This demands the use of a variety of instruments such as photo electric calorimeter, spectrophotometer, flame photometer, centrifuge, balance, distillation apparatus, pipettes etc. for the qualitative and quantitative analysis.

**Photometry** is the most common analytical technique used in clinical biochemistry. The principle of photometry is based on the physical laws of radiant energy or light. In this method, the intensity absorbed transmitted or reflected light is measured and related to the concentration of the test substance.

Photometric principles are applied in several kinds of analytical measurements.

1. Measurement of absorbed or transmitted light:  
Colorimetry, spectrophotometry & Turbidometry.
2. Measurement of emitted light:  
Flame photometry, Fluorometry.

### Colorimeter

Based on Beer-Lamberts law, Colorimetry involves measurement of colour produced by an analyte whose concentration is unknown (Test) and its comparison with that produced by known amount of substance (Standard) and intern its concentration.

*The part, use and working of Colorimeter is discussed and familiarised in the module 1 of the course.*

**Standard formula used in the Colorimetry is:**

Concentration of analyte in deciliter:

$$= \frac{\text{Absorbance (O.D) of Test}}{\text{Absorbance (O.D) of Standard}} \times \frac{\text{Concentration of Standard}}{\text{Amount of Sample}} \times 100$$

Spectrophotometer has same working principle of Colorimeter but in spectrophotometer, prism or diffraction grating is used as monochromator which provides narrower wavelength and enables special measurements in ultra violet and infrared regions.

### **Flame Photometer**

Flame photometry works on the fact that the compounds of the alkali and alkaline metals can be thermally dissociated in a flame and that some of the atoms produced will be further excited to a higher energy level. When these atoms return to the ground state they emit radiation which lies mainly in the visible region of the spectrum. Each element will emit radiation at a wavelength specific for that element. such as Sodium (Na) 589 Yellow, Potassium (K) 766 Violet. Light emitted is proportional to sample concentration. The light emitted by the element at the characteristic wavelength is isolated by an optical filter and the intensity of that light measured by a photo-detector, and then an electrical signal can be obtained proportional to sample concentration. Such an electrical signal can be processed and the readout obtained in an analogue or digital form. Flame photometer is used for determining electrolyte concentration in serum samples.

### **Centrifuge**

Centrifuge is an essential equipment of every medical laboratory to separate solid particles from a liquid suspension by means of centrifugal force. Centrifuge is required for the preparation of serum and plasma, removing precipitates etc. in the clinical biochemistry tests

### **Water Bath**

A water bath is an instrument where water is heated and the set temperature is maintained at a constant level. It is used to incubate liquid substances. Chemical tests react best at a specific temperature. Many tests react at room temperature (18 to 22°C) and others require a specific temperature as body temperature (35 to 37°C). Such procedural requirements are met by using water bath. When the reactants in

tubes are placed in a water bath, the water surrounding the tubes warms the substances inside the tube and it takes the same temperature as the water.

### **Incubator**

Incubators also used for the incubation purposes. Incubation at controlled temperature is required for clinical biochemistry analysis. The incubator is kept at a specific temperature (usually at 37°C). When tubes are kept inside the incubator, they take the temperature of the incubator. The appropriate temperature is obtained by means of temperature regulator and is maintained by a thermostat. This permits a more accurate temperature control.

*The parts, use and working of Centrifuge, Water bath and Incubator are discussed and familiarised in module 1 of the course.*

### **Distillation and Deionisation apparatus**

The quality of water used in the laboratory is very crucial. In medical laboratory work, water of an appropriate quality and quantity is required for the preparation of Standard solutions, buffers and controls, Reagents for rinsing of cleaned glass and plastic wares, cuvettes, etc. All water used in medical laboratory should be free from substances that could interfere with the tests being performed.

Depending on the requirements, available facilities and quality of the laboratory's water supply, the following instruments can be used to obtain water of adequate purity and quality.

Water distilling apparatus is an instrument that is used to purify impure water by a process known as distillation. Distillation is a process by which impure water is boiled and the steam produced is condensed on a cold surface (condenser) to give chemically pure distilled water that is water from which non-volatile organic and inorganic materials are removed. Distillation does not remove dissolved ionized gases such as ammonia, carbon dioxide, and chlorine. Distilled water should be clear, colorless and odorless.

Deionizer is an apparatus used to produce ion free water. Deionization is a process in which chemically impure water is passed through anion and cation exchange resins to produce ion free water. Deionized water has low electrical conductivity, near neutral pH and is free from water-soluble salts but is not sterile.

### **3.3.3 Blood Glucose Estimation**

Sugars are carbohydrates which are widely distributed in animals and plants, Glucose is the main sugar in the body and serves as chief fuel providing constant energy for

all activities. The blood glucose concentration remains constant in a normal healthy individual and it ranges from 80-120 mg%. Many hormones such as insulin & glucagon etc plays key role in the regulation of blood sugar. Hyperglycemia is condition of increased blood glucose level and hypoglycemia denotes decreased glucose level.

Diabetes Mellitus is a chronic disease due to disorder of carbohydrate metabolism, cause of which is either deficiency or diminished level of insulin resulting in hyperglycemia & glycosuria (presence of glucose in urine). Diabetes is a multisystem effected disorder involving many organs and systems. Symptoms of the disease include frequent urination (Polyuria), Excessive thirst (Polydypsia) and Increased hunger (Polyphagia) etc.

There are two type of diabetes (a) Juvenile Diabetes (Type I Diabetes or Insulin dependent) (b) Adult onset diabetes (Type II diabetes or Non Insulin dependent.

Diabetes is considered to be a lifestyle disorder, A number of lifestyle factors are known to be important for the development of diabetes mellitus type 2 including obesity, physical activity, diet, stress, and urbanization. Excess body fat, number of dietary factors such as sugar sweetened drinks and the type of fat in the diet appear to play a role.

Long term complications of Diabetes develops gradually, prolonged high level of blood glucose is the main risk factor which increases complications like disabling or even life threatening. The possible complications includes Nerve damage (Neuropathy), Kidney damage (Nephropathy), Eye damage (Retinopathy), Foot damage, Skin diseases, Diabetic coma etc.

### **Blood Glucose Estimation**

Blood sugar estimation is done for the diagnosis and management of diabetes and it is one of the routine analytical tests done in a clinical laboratory. There are various methods available for its estimation. previous methods like Folin Wu methods were based on the reducing property of glucose, so it may not give true value, newer methods like GOD POD Method and Hexokinase methods are enzymatic and specific, provides true glucose value.

Glucose - Oxidase method is one of the specific and most routinely employed estimation method of blood glucose determination.

### **Sample used**

For the estimation of glucose blood, serum or plasma sample are used. Whole blood, and arterial blood values are higher than venous values. Now a days serum

or plasma samples are preferred for the estimation.

Various types of samples collected for blood sugar are:

a) Fasting blood Sugar (FBS):

The blood sample is collected after the patient fasts for 12 hours or overnight.

b) Post-Prandial Blood Sugar (P P B S)

Blood is collected 1½ -2 hours after the intake of rich carbohydrate meal.

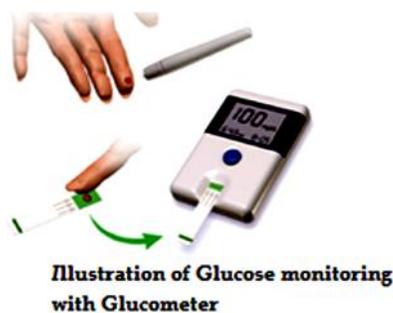
c) Random Sample (RBS)

Blood is collected at any time, regardless the time of food intake.

The plasma separated from blood sample collected in a tubes containing sodium fluoride (Na F) and potassium oxalate mixture is an ideal sample for blood glucose estimation, since Na F prevents the loss of glucose in sample by preventing glycolysis in RBC's by inhibiting the enzyme 'enolase'.

### Glucometer:

Glucometer is point of care of testing device for determining the approximate concentration of glucose in the blood. It is commonly used for home blood glucose monitoring by people with diabetes mellitus or hypoglycemia. A small drop of blood, obtained by pricking the skin with a lancet, is placed on a disposable test strip that the meter reads and uses to calculate the blood glucose level. The meter then displays the level in units of mg/dl . Most of the glucometers are based on the electrochemical technology or colour reflectance principle.



### Glucose Tolerance Test

Glucose Tolerance is defined as the capacity of the body to tolerate an extra load of glucose. Normally the blood glucose level remains relatively constant the fasting being 70-110mg%. The glucose returns to normal within 2hours after the intake of Glucose or meal. The oral G.T.T. is used to diagnose pre diabetes and Diabetic conditions.

A fasting blood sample is drawn and patient is given a measured dose of glucose solution (75 gm in 200-250ml water). Blood and urine samples are collected at half

an hour interval up to 2½ hours and sugar is estimated. The result obtained is plotted on a graph with times on the X axis and blood glucose values on Y axis.

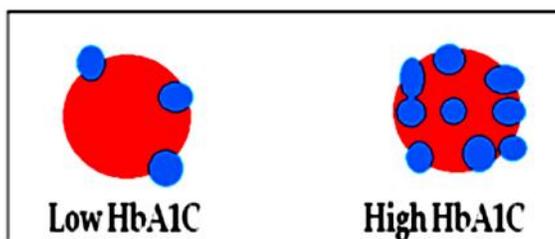
In normal glucose tolerance, the fasting sugar value will be within normal level. Peak value will be seen in 1 hour and none of the urine samples show the presence of sugar. Decreased tolerance obtained in diabetes, shows fasting sugar above normal, peak values exceeds renal threshold level and sugar may be present in urine samples.

### Glucose challenge test (GCT)

GCT is done to evaluate the body response of a patient to an immediate increase in the blood sugar value. In GCT, the patient is given 50gm of glucose in 200-250ml water and blood sample is collected after 1 hour for glucose estimation. Normal individuals metabolise the amount of glucose and maintain the normal glucose level. GCT is done during pregnancy to screen for gestational diabetes.

### Glycated hemoglobin (HbA1C)

Haemoglobin inside erythrocytes undergoes a non enzymatic chemical reaction with glucose. HbA1c concentration has been suggested as a reliable measure of the degree of metabolic control in diabetic patients. It



provides an assessment of average blood sugar control over the last 3 months.

In people without diabetes, the normal range for the HbA1c test is between 4% and 5.6%. HbA1c levels between 5.7% and 6.4% indicate increased risk of diabetes, and levels of 6.5% or higher indicate diabetes. Chromatographic method, latex immune assay or enzymatic assay are employed in HbA1c tests.

### 3.3.4: Renal function tests

The main function of the kidney is excretion of water soluble waste products from our body. The kidney has various filtration, excretion and secretory functions. Derangement of any of these function would result in either decreased excretion of waste products and hence their accumulation in the body or loss of some vital nutrient from the body. Based on the level of these excretory products and nutrients in the urine as well as in blood can make an accurate calculation to interpret the efficiency of the kidney to undertake its various functions. The different tests used to assess the renal function include various biochemical tests in blood, urine analysis, concentration dilution tests, clearance test etc.

Renal function test includes a group of tests to evaluate the function of kidney which includes S.Creatinine, Blood Urea, S.Uric acid, Serum electrolytes such as  $\text{Na}^{++}$ ,  $\text{K}^{++}$ , total protein and Albumin.

Non protein substances are nitrogen containing compounds present in the blood. They are Urea, Uric acid, Creatine & Creatinine etc. their increase in the blood level usually indicate impaired renal function.

Urea constitutes 50% of total NPN and is the end product of the protein metabolism. It is synthesized in the liver from the ammonia produced by the catabolism of amino acids. It is transported by the blood to the kidneys from where it is excreted. Average normal urea concentration is 15-40 mg/dl. Increased levels are found in renal diseases, urinary obstructions, shock, congestive heart failure and burns. Decreased levels are found in liver failure and pregnancy. Different urea estimation methods include diacetyl monoxime (DAM) method, enzymatic methods using urease etc. Berthlot method is usually employed for the determination of urea.

Creatinine, a reliable indicator of renal function is normally present in blood in trace amounts and its normal range is 0.6-1.2 mg/dl. Creatinine concentration is remarkably constant and it is the measure of glomerular filtration. Methods of Serum Creatinine estimation are Jaffe's (alkaline picrate) method and creatininase method,

Uric acid is the end product of purine metabolism. Its normal range in female is 2.5-6.0 mg/dl, and in male is 3.4-7.0 mg/dl. Increase of uric acid in the serum plasma or urine can be due to the over production of purine containing molecules or due to insufficient excretion. The concentration of uric acid is increased in various renal diseases, with increased cell lysis in the presence of tumors, leukemia etc. Prolonged elevation of the concentration leads to gout. Specific method of S.Uric acid estimation is uricase method.

Clearance tests are used to express the rate of excretion of a given substance by the kidney. It is the measure of glomerular function. Clearance of any substance is defined as the volume of blood or plasma which contains the amount of substance which is excreted in the urine in one minute. Urea and Creatinine clearance tests are commonly employed.

Determination of Cystatin C is a novel marker of Glomerular filtration. Cystatin C is a low molecular weight protein synthesized by all nucleated cells filtered at the glomerulus.. Because of its multiple advantages, cystatin C is considered a superior marker for determining GFR. Cystatin C is determined by Fluorescent and enzymatic immuno assays or Nephelometric assays. The reference range is 0.8 - 1.03 mg/l.

A urine albumin concentration ranging 30-300 mg/24 hr is called micro albuminuria. Detection of urine micro albumin is considered as a reliable marker for prediction of later development of kidney disease in diabetes. Immuno turbidometric methods are commonly employed for the detection of urine microalbumin.

### 3.3.5 Liver function tests

Liver function tests are a group of tests done to assess the function of liver, which helps to determine the presence, type of liver disease and its progression. LFT includes the following tests S. bilirubin, Total protein, S. Albumin, Globulin, ALT, AST & ALP.

Bilirubin is formed in the reticuloendothelial system during the degradation of erythrocytes. The heme portion from hemoglobin and from other heme containing proteins are removed, metabolized to bilirubin, and transported as a complex with serum albumin to the liver. In the liver, bilirubin is conjugated with glucuronic acid for solubilization to form conjugated or direct bilirubin for subsequent transport through the bile duct into the digestive tract where it is metabolized by bacteria to a group of products collectively known as stercobilinogen. Total bilirubin is the sum of the conjugated and unconjugated fractions.

Increase in bilirubin causes yellowish discoloration of skin and mucous membrane and is called Jaundice. Jaundice is classified into three groups; haemolytic (Pre hepatic) Jaundice, Infectious( Hepatic) and Obstructive( Post Hepatic jaundice).

Pre-hepatic diseases such as hemolytic disease or liver diseases resulting in impaired entry, transport or conjugation within the liver causes elevation of unconjugated (indirect) bilirubin. Total Bilirubin is elevated in conditions causing obstruction of the bile duct, hepatitis, cirrhosis, in hemolytic disorders and several inherited enzyme deficiencies.

Based on the reaction of bilirubin with diazotized suphanilic acid (Van den bergh reaction), different Bilirubin estimation methods are available. Malloy -Evelyn method is the commonly employed method used for bilirubin determination. Normal Serum bilirubin Total is 0.2- 1.0 mg% and direct bilirubin value is less than 0.5 mg%.

Serum contains a large variety of proteins. Albumin and the various globulins constitute the bulk of the total amount of proteins present in serum. Normal serum total protein concentration is 6-8 gm%, The values are lower in neonates, rise gradually in infancy and reach the adult levels in early childhood. The level decreases in pregnancy.

An increase in serum total proteins occurs in dehydration due to haemoconcentration. An increase may also occur in multiple myeloma, chronic infections, chronic liver disease and autoimmune diseases.

A decrease in serum total proteins may result from heavy losses of proteins in urine as in nephrotic syndrome. A decrease may also occur in protein malnutrition and in shock, burns, hemorrhage etc. due to haemodilution. Increased breakdown of proteins, as in hyperthyroidism, un-treated diabetes mellitus, etc. may also lower the level of proteins in serum. The most widely used method of total protein is still the biuret method. (The name derives from the reaction between biuret and cupric ions in an alkaline medium.

Albumin comprises about 50% of the total protein concentration. normal serum albumin concentration is 3.5-4.9 gm%, different method of albumin estimation includes salting out and dye binding techniques. Dye binding method 'BCG method' is commonly employed for the albumin estimation.

Globulins are estimated by subtracting albumin value from total protein value. Normal serum globulin concentration is 2-3.5 gm%. The normal Albumin/Globulin (A/G) ratio is 2:1. The A/G ratio may be used as an indicator of liver diseases, it may be decreased or reversed in response to a low albumin or to elevated globulins seen in liver disease.

As a centre of metabolic activity, Liver is rich in various enzymes such as transaminases and phosphatases. In liver diseases the destruction of liver cells cause the release of these enzymes, with consequent rise in their values in blood. Serum levels of Aspartate transaminase (AST/SGOT), alanine transaminase (ALT/SGPT) & Alkaline phosphatase (ALP) are increased in liver diseases due to damage to hepatic cells or due to obstruction in the excretion of bile. The ALT value is higher than AST in liver diseases. Detection techniques of transaminases include various colorimetric, spectrophotometric, chemiluminescence and fluorescence techniques. Normal value is 5 to 40 U/l for AST and 5 to 35 U/l for ALT and 20 to 140 IU/L for ALP.

### 3.3.6 Lipid Profile

Lipid profile or lipid panel is a panel of blood tests that serves as an initial broad medical screening tool for abnormalities in lipids, the lipid profile typically includes:

- Total cholesterol
- Triglycerides
- High-density lipoprotein (HDL)
- Low-density lipoprotein (LDL)

Using these values, a laboratory may also calculate: VLDL

LDL & VLDL values are usually calculated by using the Friedewald's equation:

$$\text{VLDL} = \frac{\text{Triglycerides}}{5}$$

$$\text{LDL} = \text{Total cholesterol} - (\text{HDL} + \text{VLDL})$$

The blood samples for lipid profile should be collected only after 10-12 hours of fasting.

Cholesterol is found in all body cells. The liver makes all of the cholesterol the body needs to form cell membranes and to make certain hormones. The determination of serum cholesterol is one of the important tool in assessing the risk in arteriosclerosis and in the diagnosis of familial and non familial lipemia. High blood cholesterol is one of the major risk factors for heart disease.

Previously estimation of serum. Cholesterol is based on colour reactions such as Lieberman burchard reaction etc. specific enzymatic method such as CHOD PAP Method is now employed for the determination of Serum Cholesterol.

Serum Cholesterol varies from 150-200 mg% in healthy young adults, increase in cholesterol usually regarded as a risk factor in the development of heart disease. An increase in serum cholesterol (hypercholesterolaemia) is found in diabetes mellitus, nephrotic syndrome, obstructive jaundice, and hypothyroidism. A decrease in serum cholesterol (hypocholesterolaemia) is found in hyperthyroidism, hepatocellular damage, anaemia (except haemorrhagic), acute infections, intestinal obstruction and terminal states of a variety of disease.

Lipoproteins includes High density lipoproteins(HDL), Low density lipoproteins(LDL), and Very low density low proteins(VLDL) are the proteins which transport fat in the blood stream. HDL is known as 'Good Cholesterol' since it carries cholesterol form peripheral tissues to liver for excretion. Optimum level of HDL cholesterol is above 60 mg%. HDL Cholesterol below the level of 40mg% may indicate the increased risk of coronary disease. LDL carries cholesterol and triglycerides from liver to extra hepatic tissues leading to atherosclerosis. Hence LDL is termed as 'Bad Cholesterol'. The desirable level of LDL is less than 100 mg%

Triglycerides are esters of fatty acids with glycerol are the most common type of fat in the body, that mostly come from food. High levels of blood triglycerides are often found in people who have high cholesterol levels, heart problems, are overweight or have diabetes. Its desirable level is below 150 mg%.

In addition to serum Total cholesterol, the measurement of HDL, LDL and Triglycerides provide valuable information for the risk assessment of coronary heart diseases.

### 3.3.7: Other parameters of Diagnostic importance

Clinical Biochemistry is the rapidly advancing areas of a Clinical laboratory having about 700 different tests available today. The marked increase in the number and availability of laboratory diagnostic procedures has helped in the solution of clinical problems. Individual laboratory tests are rarely ordered and reported singly; usually combinations of lab tests are used. Biochemical investigations like blood glucose, LFT and RFT are done routinely for most patients were as special biochemical profiles such as TFT, Infertility profile, electrolytes, Cardiac and tumour markers are now increasingly requested by the physician which helps them in screening various endocrine malfunctions and cancers.

Determination of body fluid concentrations of the four major electrolytes ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ , and  $\text{HCO}_3^-$ ) is commonly referred to as an "electrolyte profile." Sodium and Potassium are commonly ordered at regular intervals to monitor treatment of certain conditions, including high blood pressure (hypertension), heart failure, lung diseases, and liver and kidney disease .

Reference level:- Sodium: 135-145 M Eq/l Potassium: 3-5.0 M Eq/l

S.Sodium and Potassium are determined usually together by flame photometry, Ion Sensitive Electrode (ISE) or Spectrophotometric methods.

#### **Clinically important minerals**

Determination of Serum calcium and phosphorus levels help to diagnose disorders of mineral metabolism. Normal calcium level is between 8.5-10.5 mg/dl. Decreased calcium level are found in Rickets, Hypoparathyroidism and Renal failure. Increased calcium level is seen in Hyper parathyroidism and in bone tumours .

Normal serum concentration of phosphorus is 2.5- 4.5 mg/dl. Increased level are found hypoparathyroidism, and renal failure. Decreased levels are seen in hyper parathyroidsism, Rickets and Vitamin D deficiency.

#### **Clinically important hormones**

Thyroid hormones such as Thyroid-stimulating hormone (TSH, thyrotropin), Thyroxine (T4), Tri iodothyronine (T3). are estimated to check the function of the thyroid. Thyroid function tests (TFT) is a collective term used for these hormones. TFTs may be requested if a patient is thought to suffer from hyperthyroidism

(overactive thyroid) or hypothyroidism (underactive thyroid), or to monitor the effectiveness of either thyroid-suppression or hormone replacement therapy.

Sex Hormones like Follicle-stimulating hormone (FSH), Prolactin (PRL), Progesterone, Estrogen, Free and total testosterone, Luteinizing hormone (LH) are estimated to evaluate endocrine function. These hormones included in the infertility profile can aid in determining causes of infertility.

### **Clinically important Enzymes**

Different forms of acid phosphatases originated from different organs such as prostate, liver, bone and Red cells. are found in blood. Determination of prostatic fraction of acid phosphatase is useful in the diagnosis of prostatic cancer.

The blood amylase test is used to help diagnose and monitor acute pancreatitis. The reference range for Serum amylase is 40-140 U/L

Gamma glutamyl transferase (GGT) is an enzyme of hepatic origin is often included in the liver function tests. Normal serum level of GGT is 10- 50 U/L. Elevated levels are found in liver diseases, pancreatic diseases and chronic alcoholism.

### **Cardiac markers:**

Cardiac biomarkers are substances that are released into the blood when the heart is damaged or stressed. Measurements of these biomarkers are used to diagnose acute coronary syndrome (ACS) and cardiac ischemia.

Currently using cardiac markers includes

- Troponins (Troponin I & Troponin T)
- CK MB,
- Lactate dehydrogenase (LDH),
- Aspartate serum transaminase (AST)
- Myoglobin
- Natriuretic peptide.

### **Tumour markers**

Tumour markers are substances, often proteins, that are produced by the cancer tissue itself or sometimes by the body in response to cancer growth. Because some of these substances can be detected in body samples such as blood, urine, and tissue, these markers may be used, along with other tests and procedures to help to diagnose some types of cancer, predict and monitor a person's response to certain treatments and detect recurrence. Tumour markers can be determined in serum or rarely in urine or other body fluids often by immunoassay.

Commonly used tumour markers include

- Alfa fetoprotein (AFP),
- Carcinoma Embryonic antigen (CEA),
- Prostate specific antigen (PSA),
- CA-125,
- CA-19.9
- Beta Human chorionic gonadotropin ( $\beta$ hCG)

### **Blood Gas Analysis:**

Arterial blood gas (ABG) analysis test measures parameters such as pH,  $P_{O_2}$ ,  $P_{CO_2}$  &  $HCO_3^-$  in the blood for the Clinical management of acid-base imbalance, respiratory and metabolic disorders.

### **3.3.8 Quality control in biochemistry**

Nowadays the clinicians mainly depend on the laboratory results for the diagnosis and treatment of diseases. Quality results at right time gains great value in a laboratory practice. Various quality control measures are adopted in medical laboratory to reduce pre analytical, analytical and post analytical errors.

Quality assurance defined by WHO is a Total process whereby the quality of lab reports can be guaranteed. Quality control programme involves various steps taken both inside and outside the laboratory in order to ensure quality of results given

#### **Internal quality control**

This is based on monitoring the Biochemistry test procedures that is performed within the laboratory. It includes measurement of specially prepared materials and repeated measurements on routine specimens as well as statistical analysis of day by day data obtained from the test which has been routinely carried out. There is thus continuous evaluation of the reliability of the work of the laboratory. Hence IQC primarily check the precision of lab work.

#### **External quality assessment (EQAS)**

This is the evaluation of laboratory procedures by an outside agency on specially supplied samples. Analysis of performance is retrospective. The objective is to achieve comparison between lab and between method compatibility, but this doesn't guarantee accuracy unless the specimens have been assayed by the reference lab alongside a reference preparation of known value. Schemes are usually organized on a national or regional basis. Hence, EQA is mainly concerned with analytical part of the test.

**Common terms used in Quality control includes**

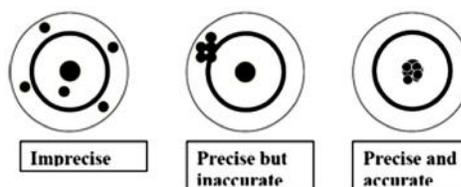
Accuracy refers to the closeness of the estimated value to that of true value.

Precision refers to the closeness of the result on repeated analysis,

Specificity is the ability of an analytical method to determine solely the analyte it is required to measure.

Sensitivity is the ability of an analytical method to detect smallest quantities of the measured analyte.

Analytical methods require calibration, the process of relating the value indicated on the scale of the measuring device to the quantity required to be measured. Calibration is done using standard, the solution with which the sample is compared to arrive at the result. Standard solutions refer to the known amount of a substance in a solution in which its concentration is expressed in terms of moles or in weights per unit volume.



- **Mean:** Refers to the arithmetic average of a set of values. A measure of central tendency of the distribution of a set of replicate results. Often abbreviated by an x with a bar over it.
- **Standard deviation-** indicates the average error, used to define how much the different value in a series of similar determinations differ from one another on a same sample.

$$S = \sqrt{\frac{\sum (x_n - \bar{x})^2}{n - 1}}$$

X - Individual determinations

$\bar{x}$  - Mean of Determinations

n : No of Determinations

- **Coefficient of variation (CV)/**The relative standard deviation, i.e., the standard deviation expressed as a percentage of the mean [CV=100(s/x)].
- **Bias :** A systematic difference or systematic error between an observed value and some measure of the truth. Generally used to describe the inaccuracy of a method relative to a comparative method in a method comparison experiment

**Types of errors:**

Two major types of errors may occur in a laboratory.

- **Random error:** that arise due to in adequate control on pre analytical variables - patient identity, sample labelling, sample collection, handling and transport etc.

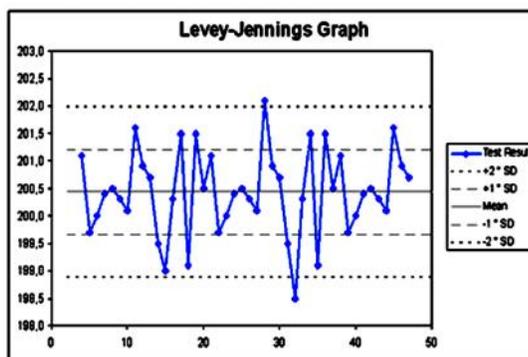
$$\text{Individual measurement minus mean } (X - \bar{X})$$

- **Systemic error:** That occur due to inadequate control on analytical variables eg. due to error in calibration, impure/unstable calibration material, unstable reagent blanks etc.

Mean minus true value.

### Levey-Jennings (LJ) chart

Levey-Jennings chart is the most important control chart in laboratory quality control. It can be used for internal and external quality control as well. It detects all kinds of analytical errors (random and systematic) and is used for the estimation of their magnitude. On the x-axis the date and time, or more



usually the number of the control run, are plotted. A mark is made indicating how far off the actual result was from the mean (which is the expected value for the control). Lines run across the graph at the mean, as well as one, two and sometimes three standard deviations either side of the mean. This makes it easy to see how far off the result was.

### 3.3.9 Automation and Recent advances

During the past few year, in clinical biochemistry there has been a considerable increase in clinical demand for laboratory investigations. When the volume of work increased, there arose a need for work simplification. Mono step methods are introduced to replace multistep cumbersome and inaccurate methods like Folin-Wu's blood sugar determination. The efficiency of monostep methods was further increased by the introduction of automatic dispensers and diluters. For the common test like blood glucose, blood urea etc., however, most large laboratories found this approach still inadequate to deal with work load and instruments designed to handle the whole analytical process in mechanized fashion have become common place in last decade.

**Automation** refers to the mechanization of manual techniques with the minimal involvement of an analyst or technician. The availability of automated instruments enables laboratories to manage much larger workloads without comparable increases in staff. Automation simply being used to assist the laboratory technologist in test performance, but it now includes

- (1) Processing and transport of specimens,
- (2) Loading of specimens into automated analyzers,
- (3) Assessment of the results of the performed

Reduction in the variability of results, errors of analysis and the improved reproducibility gained by automation has led to a significant improvement in the quality of laboratory tests. Autoanalyser replaces the steps of pipetting, protein free filtratration, manual incubation etc. with automated devices and increases the accuracy and precision of the methods. Following are the different types of autoanalysers used in clinical chemistry laboratories.

### Types of analyzers

**Semi-auto analyzer:** It is a partial automation technique Here, the samples and reagents are mixed and read manually. Calculation and reporting is done by the instrument.



Semiautoanalyser



Fully automatic analyser

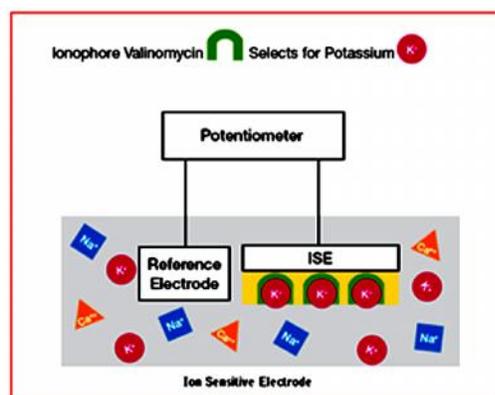
**Fully Auto Analyser :** Here all the steps including pipetting and mixing of sample and reagent, incubation, reading, calculation and reporting is done by the instrument. Fully auto analyser can be of different types.

**Batch analyzer:** . One parameter is estimated at a time enabling one batch of a specific test to be automatically conducted. The next parameter is estimated only after completion of one.

**Random Access autoanalyzers:** These analyzers can store more than one reagent. Samples are placed in the machine and the computer is programmed to carry out any number of selected tests on each sample. Priority can be given to test any sample or any test without any specific order. Hence they are also called STAT (Short Turn Around Time) Analysers. They can perform single test, profile pattern or emergency tests.

### Electrolyte Analyser

Electrolyte analysers are the latest automated instrument used for the estimation of electrolytes such as sodium ( $\text{Na}^+$ ), potassium ( $\text{K}^+$ ) etc. in place of flame photometer. The instrument uses Ion selective electrodes and is based on the potentiometric method of ion



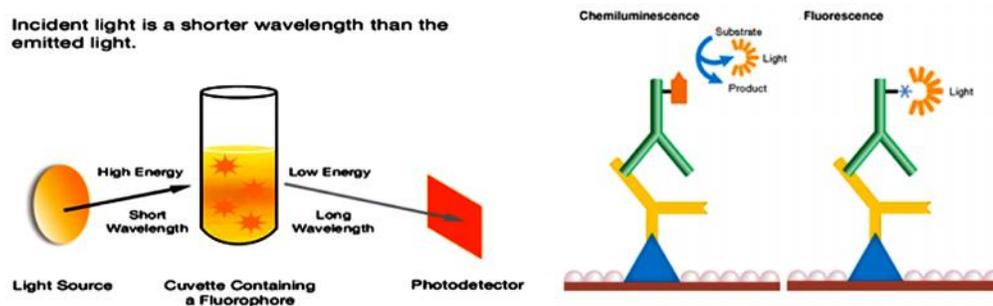
measurement. ISEs use membranes with very selective permeability or sensitivity to the analyte ion's size and charge. The membranes may have pores that limit the size of ions that may enter the membrane. They may be impregnated with chemicals,

called ionophores, which allow only selected ions to be sensed by the electrode. For example, a polymer membrane that incorporates an ionophore called valinomycin is highly selective for potassium with little to no response to physiologic concentrations of other ions like sodium, calcium or magnesium.

### Advanced Assay Techniques

**Chemiluminescent immunoassay (CLIA)** is one of the latest trends in diagnosis. Chemiluminescence is the light emission produced during a chemical reaction. In a chemiluminescent immunoassay, a chemiluminescent molecule is used as an indicator label to detect and quantify immunological reactions. Isoluminol and acridinium esters are examples of chemiluminescent labels. Oxidation of isoluminol by hydrogen peroxide in the presence of a catalyst (e.g., microperoxidase) produces a light emission at 425 nm. Oxidation of an acridinium ester by alkaline hydrogen peroxide in the presence of a detergent (for example, Triton X-100) produces a rapid flash of light at 429 nm. The concentrations of analytes are determined by measuring these wavelengths.

Chemifluorescence assay involves the enzymatic conversion of a substrate to a fluorescent product. The enzyme cleaves a phosphate group from a fluorogenic substrate to yield a highly fluorescent product. The fluorescence can be detected using a fluorescence imager.



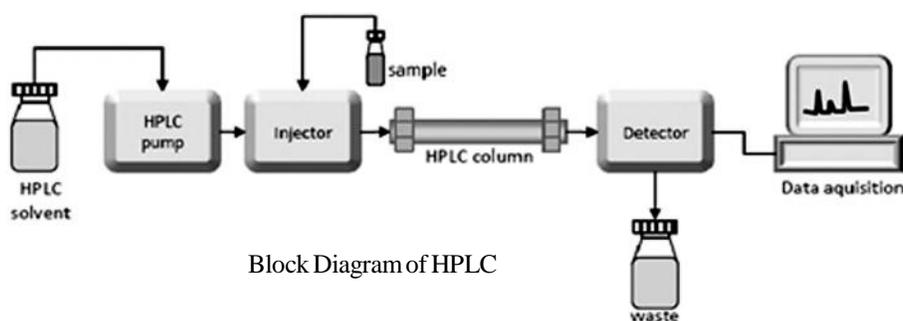
**Turbidometry:** The assay involved with measuring the amount of transmitted light. Commonly employed in the determination of the concentration of total protein in urine and CSF which contain small quantities of protein (mg/L)

**Nephelometry:** is concerned with measurement of scattered light from a cuvette containing suspended particles in a solution. Nephelometry used in the determination of immunoglobulin (total, IgG, IgE, IgM, IgA), haptoglobin, C-reactive protein, etc in serum.

**High performance liquid chromatography (HPLC)** is basically a highly improved form of column liquid chromatography. HPLC has the ability to separate, and identify compounds that are present in any sample that can be dissolved in a liquid in trace concentrations as low as parts per trillion. Instead of a solvent being allowed to drip through a column under gravity, in HPLC a sample mixture or analyte is forced through a solvent (known as the mobile phase) at high pressure through a column with chromatographic packing material (stationary phase). The sample is carried by a moving carrier gas stream of helium or nitrogen.

Clinical applications of HPLC

1. Urine analysis, antibiotics analysis in blood.
2. Analysis of bilirubin, biliverdin in hepatic disorders.
3. Detection of endogenous Neuropeptides in extracellular fluid of brain etc.



Block Diagram of HPLC

**Point-of-care testing (POCT)** is the testing at or near the point of care ie at the bedside, this contrasts with the historical pattern in which testing mostly confined to the medical laboratory, POCT brings the test conveniently and immediately to the patient. POCT utilizes dry chemistry principles, uses easy-to-use membrane-based test strips, enclosed by plastic test cassettes and often accomplished through the use of portable, and handheld instruments e.g., blood glucose meter. POCT includes blood glucose testing, blood gas and electrolytes analysis, rapid coagulation testing (PT/INR), rapid cardiac markers diagnostics, drugs of abuse screening, urine strips testing, pregnancy testing, faecal occult blood analysis, food pathogens screening, haemoglobin estimation, infectious disease testing and cholesterol screening etc.

## DETAILING OF PRACTICALS

### Cleaning of used Glassware for biochemical analysis

#### Procedure:

1. Used glassware given should be washed with water immediately after use
2. Soak in mild detergent solution for considerable period of time.
3. Wash thoroughly with running tap water
4. Rinse with distilled water.
5. Allowed to dry in Hot air oven at 80°C.

### Operation of Colorimeter, Centrifuge, Water bath, incubator,

To operate an instrument carefully under the supervision of teacher, prepare a record log of the working, Care and maintenance of the instruments.

### Blood Sugar estimation by GOD POD method

#### Principle:

Glucose oxidase catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide. This  $H_2O_2$  is broken down to water and oxygen by a peroxidase, the oxygen formed will bind with 4 amino antipyrine and alcohol to form red colour. The amount of red colour formed is directly proportional to the amount of glucose present in the sample, which can be measured colorimetrically.

Requirements:

Glucose Reagent

Glucose standard (100mg%)

Sample : Plasma/Serum

#### Procedure:

1. Take 3 test tubes and label as T,S& B
2. Pipette the reagents as given in the table below

	<i>Test (T)</i>	<i>Standard(S)</i>	<i>Blank (B)</i>
Reagent	1 ml	1 ml	1ml
Sample	10 $\mu$ l	-	-
Standard solution	-	10 $\mu$ l	-
D.Water	-	-	10 $\mu$ l

3. Mix and incubate for 10 min at 37°C or 15-20 min at room temperature (15-25°C), Read the absorbance (A) of the Test and standard, against the Blank using Green filter(540nm).

4. Calculation:

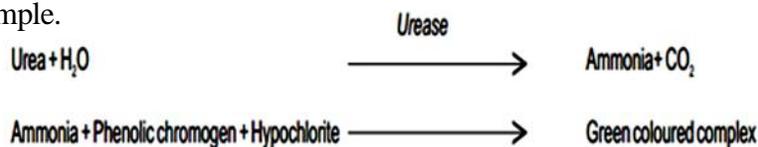
$$\text{mg/dl glucose in the sample} = \frac{\text{Abs of Sample}}{\text{Abs of Standard}} \times 100$$

Normal value: 80-120 mg%

### Estimation of Urea by Berth lot method.

#### Principle:

Urease hydrolyzes urea to ammonia and CO<sub>2</sub>. The ammonia formed further reacts with a phenolic chromogen and hypochlorite to form a green coloured complex. Intensity of the colour formed is directly proportional to the amount of urea present in the sample.



#### Requirements:

1. Buffer Reagent
2. Enzyme Reagent
3. Chromogen Reagent
4. Urea Standard (40 mg/dl)

Working Enzyme Reagent: a working enzyme reagent may be made by pouring 1 bottle of Enzyme Reagent into 1 bottle of Buffer Reagent.

Sample used: Serum/ plasma

#### Procedure:

1. Take 3 test tubes and label as T, S & B
2. Pipette the reagents as given in the table below

	<i>Test (T)</i>	<i>Standard(S)</i>	<i>Blank (B)</i>
Enzyme Reagent	1 ml	1 ml	1ml
Sample	10µl	-	-
Standard solution	-	10µl	-
D.Water	-	-	10µl
Mix well and incubate for 5 mins. at 37° C or 10 mins. at R.T. (25° C)			
Chromogen Reagent	0.2 ml	0.2 ml	0.2 ml

Mix well and incubate for 5 minutes or 10 minutes at R.T. (25o C). Measure the absorbance of the Standard and Test Sample against the Blank at 578 nm, within 60 minutes.

$$\text{Calculation: Urea in mg/dl} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Std.}} \times 40$$

Normal Value; 15-40 mg/dl

### Estimation of S.Creatinine by Jaffe's Kinetic method

#### Principle:

Creatinine reacts with alkaline picrate to produce a red coloured complex. The intensity of red colour formed during fixed time is directly proportional to the Creatinine concentration.

Requirements :

1. Picric acid Reagent
2. Alkaline Buffer Reagent
3. Standard Creatinine (2mg%)

Working reagent preparation: Mix equal volumes of Picric Acid Reagent (1) & Alkaline Buffer Reagent(2). Working Reagent is stable for 10 days in a Brown Bottle at room temperature.

Sample used : Serum, Plasma or Urine can be used.

#### Procedure:

1. Take 2 test tubes and label as T& S
2. Pipette the reagents as given in the table below

	Test(T)	Standard(S)
Working reagent	1.0 ml	1.0 ml
Sample	100µl	-
Standard solution	-	100µl

Mix well and allow it to stand at R.T. for 5 mins. Read the initial absorbance for standard(AS0) and Test (AT0)against distilled water at 520 nm (green filter) after exactly 30 seconds. Read another absorbance of standard (AS1) and Test (AT1) against distilled water at 520 nm (or) green filter exactly after 60 seconds later.

$$AS = AS0 - AS1$$

$$AT = AT_0 - AT_1$$

$$\text{Serum Creatinine in mg/dl} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Std.}} \times 2$$

Normal Value; 0.6-1.2 mg/dl

### Estimation of S.Uric acid by Uricase method

#### Principle:

Uricase transforms Uric acid into Allantoin, Carbon dioxide (CO<sub>2</sub>) and Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). By the action of Peroxidase and in the presence of DHBS and 4-Aminoantipyrine, Hydrogen peroxide gives a coloured product which can be measured at 520 nm. the amount coloured product formed is proportional to the uric acid concentration of the sample.

Requirements:

Reagent (R1) Phosphate buffer & DHBS (3, 5 Dichloro 2 hydroxy benzene sulphonic acid)

Reagent (R2) Uricase & Aminoantipyrine

Dissolve one vial of R2 in appropriate amount of R1.

Uric acid standard (4 mg/dl)

Sample used: Serum.

#### Procedure:

1. Take 3 test tubes and label as T, S & B
2. Pipette the reagents as given in the table below

	<i>Test (T)</i>	<i>Standard(S)</i>	<i>Blank (B)</i>
Working reagent	1 ml	1 ml	1ml
Sample	20µl	-	-
Standard solution	-	20µl	-
D.Water	-	-	20µl

Mix well and incubate at 37° c, for 10 minutes and read the absorbance using green filter Calculation:

$$\text{S.Uric acid concentration} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Std.}} \times 4$$

Normal Value; Female: 2.5-6.0 mg/dl, Male: 3.4-7.0 mg/dl

### Estimation of Serum.Bilirubin by Malloy-Evelyn method

#### Principle:

Bilirubin is reacted with diazotized sulfanilic acid and converted to a purple coloured

azobilirubin, the intensity of which is read colorimetrically. Both conjugated and unconjugated bilirubins give purple azobilirubins with diazotized acid. Conjugated bilirubin can react in aqueous solution (Direct Reaction), whereas unconjugated requires an accelerator, such as methanol (Indirect Reaction-which gives total bilirubin i.e. conjugated + unconjugated bilirubin).

### Requirements::

1. Diazo reagent : Make freshly before use the mixing 10ml of solution A and 0.3ml of solution B.  
Solution A : 1g of sulphanilic acid and 15ml of concentrated HCL per litre in water.  
Solution B : 0.5g of sodium nitrite/100ml in water. This solution should be kept in refrigerator renewed monthly.
2. Diazo Blank : 15ml of conc. HCL/litre in water.
3. Methanol
4. Bilirubin standard

Sample used: Serum

### Procedure:

1. Take 3 test tubes and label as T, D & B
2. Pipette the reagents as given in the table below

	Total (T)	Direct (D)	Blank(B)
Serum	0.2 ml	0.2 ml	0.2 ml
Distilled water	1.8 ml	1.8 ml	1.8 ml
Diazo reagent	0.5 ml	0.5 ml	-
Diazo blank	-	-	0.5 ml
Methanol	2.5 ml	-	2.5 ml
Water	-	2.5 ml	-

Mix and keep the T and B in the dark for 30 minutes and keep the test tube labeled D at room temperature for one minute.

Read the absorbances of T, D, B and Standard using green filter (540 nm) in colorimeter.

Calculation:

Concentration of total bilirubin in mg%

$$= \frac{\text{Absorbance of T} - \text{Absorbance of B}}{\text{Absorbance of Std.} - \text{Absorbance of B}} \times \text{Concentration of Std.}$$

Concentration of Direct bilirubin in mg%

$$= \frac{\text{Absorbance of D} - \text{Absorbance of B}}{\text{Absorbance of Std.} - \text{Absorbance of B}} \times \text{Concentration of Std.}$$

Indirect Bilirubin (Unconjugated bilirubin) = Total bilirubin - Direct bilirubin.

Normal Value:

Total Bilirubin: 0.2-1.0 mg%

Direct Bilirubin: 0.2-0.5 mg%

Indirect Bilirubin: 0.2-0.8 mg%

### Estimation of Total Protein by Biuret Method

#### Principle

Cupric ions in biuret reagent reacts with peptide bonds of proteins in an alkaline medium to form a purple coloured complex. The intensity of the purple colour formed is proportional to the number of peptide bonds which, in turn, depends upon the amount of proteins in the specimen.

Requirements:

1. Biuret Reagent
2. Standard protein solution (6 gm%)
3. Sample used; Serum

#### Procedure :

1. Take 3 test tubes and label as T, S & B
2. Pipette the reagents as given in the table below

	<i>Test (T)</i>	<i>Standard(S)</i>	<i>Blank (B)</i>
Working reagent	1 ml	1 ml	1ml
Sample	20µl	-	-
Standard solution	-	20µl	-
D.Water	-	-	20µl

Mix well and incubate at 37°C for 10 minutes, read the absorbance using green filter

Calculation:

$$S. \text{ Total protein concentration in gm\%} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Std.}} \times 6$$

Normal Value:

Serum total protein is 6-8 gm%

### Estimation of Serum. Albumin by BCG method

#### Principle:

Albumin binds with bromocresol green at pH 4.2 to form a greenish blue coloured complex. the amount of green colour formed is directly proportional to the amount of albumin present in the sample.

#### Requirements

1. Bromocresol green Reagent
2. Standard protein solution 4 gm%
3. Sample used; Serum

#### Procedure :

1. Take 3 test tubes and label as T, S & B
2. Pipette the reagents as given in the table below

	<i>Test (T)</i>	<i>Standard(S)</i>	<i>Blank (B)</i>
Working reagent	1 ml	1 ml	1ml
Sample	10 $\mu$ l	-	-
Standard solution	-	10 $\mu$ l	-
D.Water	-	-	10 $\mu$ l

Mix well and incubate at 37 $^{\circ}$ c for 5minutes, read the absorbance using red filter (630nm)

Calculation:

$$S. \text{ Albumin concentration in gm\%} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Std.}} \times 4$$

Normal Value:

Serum albumin is 3.5-4.9 gm%

(Globulin = Total protein - Albumin, Normal value of globulin 2-3.5gm%)

### Estimation of Serum Total Cholesterol by CHOD PAP Method

#### Principle :

Cholesterol esters are hydrolysed by cholesterol ester hydrolase to free cholesterol & fatty acids. the free cholesterol produced and pre-existing one are oxidised by cholesterol oxidase to Cholestenone-4-en-3-one and hydrogen peroxide.

Peroxidase acts on hydrogen peroxide and liberated oxygen reacts with the 4-amino phenazone to form a red coloured compound which is read at 510 nm (505-530 nm).

Requirements:

- CHOD PAP Reagent
- Cholesterol standard (200 mg%)
- Sample: Plasma/Serum

**Procedure:**

1. Take 3 test tubes and label as T, S & B
2. Pipette the reagents as given in the table below

	<i>Test (T)</i>	<i>Standard(S)</i>	<i>Blank (B)</i>
Reagent	1 ml	1 ml	1ml
Sample	10µl	-	-
Standard solution	-	10µl	-
D.Water	-	-	10µl

Mix and incubate for 5 min at 37°C or 15-20 min at room temperature (15-25°C), Read the absorbance (A) of the Test and standard, against the Blank using Green filter (540nm).

4. Calculation:

$$S. \text{ Total Cholesterol concentration in mg\%} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Std.}} \times 200$$

Normal Value; 150-200 mg/dl

**ASSESSMENT ACTIVITIES**

(For Continuous assessment)

Chart preparation showing specimens and important biochemical investigations done with them

Chart preparation showing Clinical symptoms and common complications in diabetes

Seminar on Laboratory diagnosis of Diabetes Mellitus

Assignment on Clinical relevance of RFT & LFT

Assignment on Importance of Lipid profile and various tests included

Debate on Advantages and disadvantages of Automation

### Theory Evaluation Questions

1	<p>Liver Profile and Renal Profile are group of tests commonly done in labs</p> <p>a) Name the different tests included in Liver profile ( 3 score)</p> <p>b) Write the principle, procedure calculation and normal value of any one test included in renal profile (6 score)</p>															
2	<p>Match the columns of the given table in the correct order</p> <table border="1" style="margin-left: auto; margin-right: auto;"> <thead> <tr> <th>Test</th> <th>Method</th> <th>Normal Value</th> </tr> </thead> <tbody> <tr> <td>Glucose</td> <td>Berthlot</td> <td>0.2-1.0 mg/dL</td> </tr> <tr> <td>Urea</td> <td>Evelyn Malloy</td> <td>70-110mg%</td> </tr> <tr> <td>Cholesterol</td> <td>GOD POD</td> <td>150-200 mg%</td> </tr> <tr> <td>Bilirubin</td> <td>CHOD PAP</td> <td>15-40 mg/dL</td> </tr> </tbody> </table>	Test	Method	Normal Value	Glucose	Berthlot	0.2-1.0 mg/dL	Urea	Evelyn Malloy	70-110mg%	Cholesterol	GOD POD	150-200 mg%	Bilirubin	CHOD PAP	15-40 mg/dL
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Bilirubin	CHOD PAP	15-40 mg/dL														
3	<p>Diabetes Mellitus is a disease most commonly seen among the population of Kerala. A person suspecting Diabetes comes to your laboratory</p> <p>a) Advise him the tests to be done and time of sample collection for the test</p> <p>b) Write the principle, requirements and method of a commonly used method of blood sugar estimation (5 score)</p>															
4	<p>Identify the following</p> <p>a. Reagent used for Total protein estimation</p> <p>b. Instrument used for estimation of Electrolytes</p> <p>c. Enzymatic method of cholesterol estimation</p>															
5	<p>Explain the clinical significance of the tests</p> <p>A) HbA1C B)PSA C)S.Calcium D) Microalbumin in urine (4x1=4)</p>															
6	<p>Urine examination of a patient reveals presence of puscells, RBC's, Casts and Increased Proteinuria. Doctor suspects defective function of a vital organ and suggest blood test for confirmation.</p> <p>a) Name the organ affected and suggest the tests advised.</p> <p>b) Write the procedure of any one biochemical test asked by the doctor.</p> <p>c) Write the normal value of the test. (2+4+1)</p>															
7	<p>Auto analyzers are essential for a laboratory.Prepare a comparison chart between analyzersAdd a note on the merits of automation. (4+2=6)</p>															

### EXTENDED ACTIVITIES

- Organize a Visit to observe the design, layout, safety measures & Various procedures in a Clinical Laboratory
- Visit to a speciality laboratory to see the lab automation, barcoding and LIMS
- To conduct Medical camp on diabetes detection
- Exhibition/Expo
- Prepare a Model of Ideal Laboratory
- Arrange On the Job Training in pathology and Biochemistry laboratories.

### LIST OF PRACTICAL

#### 3.1 Laboratory Management

1. Demonstration of signs and symbols used in laboratory
2. Preparation of different types of laboratory request forms
3. Preparation of different types of laboratory Report forms- Haematology, Biochemistry, Clinical pathology, Serology, Mixed forms
4. Prepare a lay out plan for a multi room laboratory
5. Preparation of models of stock registers- consumables, Non-consumable
6. Preparation of model of Order form
7. Demonstration of colour coding for biomedical waste segregation

#### 3.2 Clinical Pathology

8. Physical examination of urine.-volume, colour, specific gravity, Reaction, PH
9. Chemical examination of urine- Test for sugar(Benedict's qualitative test), Test for protein (Heat and acetic acid test), Test for Ketone bodies (Rotheras test), Test for Bile pigments (Fouchets test) and test for Bile salt(Hays test)
10. Microscopic examination of urine- Wet mount preparation of sediments, examination and reporting of sediments.
11. hCG detection in urine
12. Physical examination stool- consistency ,colour, mucus and parasites
13. Chemical examination stool-occult blood, reducing substances

14. Microscopic examination stool-saline and iodine mount
15. Physical examination of semen- Volume, colour, reaction and liquefaction time
16. Microscopy of semen-Total sperm count, motility, morphology

### **3.2 Clinical Biochemistry**

17. Estimation of Blood Glucose (GOD-POD Method)
18. Estimation of S.Cholesterol (CHOD-PAP Method)
19. Estimation of Blood Urea ( Berthlot method)
20. Estimation of S.Creatinine (Jaffe's method)
21. Estimation of S.Uric Acid (Uricase method)
22. Estimation of S.Bilirubin (Evelyn Malloy method)
23. Estimation of S.Total Protein (Biuret method)
24. Estimation of S.Albumin (BCG Method)

## Module 4

### 4.1 DIAGNOSTIC MICROBIOLOGY & HISTOTECHNOLOGY

#### Overview

Laboratory diagnosis of infectious diseases with various conventional as well as modern technologies is the main stay of Diagnostic Microbiology procedures and protocols. Culture methods are still considered as the gold standard of diagnosis. Here an attempt has been made to introduce the novel and emerging immunological and molecular techniques and their applications also.

Despite the advances in nanotechnology and nucleic acid level diagnosis, morphological characters of cells still remain as a preferred way of diagnosis in histological as well as cytological abnormalities. Information provided by a stained slide of tissue section has immense value in the present diagnosis of diseases especially that are of malignant origin. Different manual techniques that help in the preparation of specimens for histopathological and cytological studies are discussed here to introduce this special area of diagnostic laboratory to the students which may inspire them to pursue higher education in this field.

#### Introduction

The understanding of microorganisms as causative agents of infectious diseases has replaced several concepts in microbiology. The past one decade have witnessed knowledge revolutions in various branches of Microbiology. Medical microbiology is the part which touches the health and diseases of man. Diagnostic microbiology explains the various laboratory procedures involved in the process of diagnosis. It is quite cumbersome to discuss all these aspects here. In spite, all the fundamental scientific characters of clinically important microorganisms in the diagnostic point of view have been discussed here. These include laboratory diagnosis of common bacterial, protozoal and viral diseases encountered during routine laboratory practice. Importance has been given for clinical and laboratory protocols from optimal specimen collection to an accurate diagnosis.

#### Learning outcomes

##### The learner:

- defines Microbiology, to differentiate Pathogen, Commensals and type of infection
- summarizes various historical aspects of microbiology

- explains the structure of bacteria
- differentiates bacteria based on morphology, Motility & Oxygen requirement
- identifies the importance of sterilization
- categorizes different methods of sterilization
- operates Hot air oven, Autoclave & Incubator
- distinguishes Disinfectants and Antiseptics and their applications
- explains various growth requirements of bacteria
- classifies different culture media and prepare Common culture media
- explains the different inoculation techniques.
- performs the streak culture technique
- classifies different methods for the identification of bacteria
- defines Immunology and to explain different terms in immunology
- explains the structure of antibody and to classify different types of antibodies
- differentiates various Antigen- Antibody reactions and their clinical applications
- explains the Collection and transportation of different specimens
- summarizes the different methods in the specimen processing
- differentiates common bacterial diseases and identify the pathogens encountered
- explains the method of Antibiotic sensitivity test
- differentiates and perform common serological tests
- defines virology and classify viruses
- differentiates common viral diseases and the pathogen encountered
- explains common diagnostic serological tests for viral infections
- defines parasitology and explain different terms in parasitology
- differentiates intestinal and blood parasites
- identifies common blood parasites and explain their lab diagnosis
- differentiates the Causative agent, different species, host and mode of transmission of malaria
- name the different stages of Malarial parasite
- explains different methods for the diagnosis of malaria
- differentiates the disease, mode of transmission, host and nocturnal habit of filarial parasite
- explains different methods for the lab diagnosis of filariasis

- identifies common intestinal parasites and the method of examination of stool by concentration technique
- familiarizes the disease, causative agent, mode of transmission and lab diagnosis of amoebiasis
- differentiates common helminthes and explain their lab diagnosis

### 4.1.1 Introduction to Medical Microbiology

Micro-organisms or microbes are organisms that are so small (invisible to the naked eye). The study about them is called microbiology. They are single-celled or unicellular organisms, widespread in nature and are beneficial to life. But some can cause serious harm also. Microorganisms can be divided into different types like bacteria, fungi, protozoa, viruses, etc.

Medical microbiology is the study about the microbes and their role in human illness. Disease causing organisms are called Pathogens. The study also Includes microbial pathogenesis (The mechanism by which the disease is produced), epidemiology (Spreading) and immunology. It involves identification, treatment or prevention of diseases caused by bacteria, virus, and fungi.

Microbiology can be also classified based on taxonomy

Bacteriology	:	The study of bacteria.
Mycology	:	The study of fungi.
Protozoology	:	The study of protozoa.
Parasitology	:	The study of parasites.
Immunology	:	The study of the immune system.
Virology	:	The study of viruses.

### Infection

Infection is the invasion, lodgment and multiplication of organism in the tissue of host. Not all infections however results in disease. Depending on the spread of infectious disease in the community they may be classified into different types

### Classification of infections

Primary infection	:	Initial infection with organism in host.
Reinfection	:	Subsequent infection by same organism in a host (after recovery).
Superinfection	:	Infection by same organism in a host before recovery.

- Secondary infection : When in a host whose resistance is lowered by pre-existing infectious disease, a new organism may set up in infection.
- Focal infection : It is a condition where due to infection at localized sites like appendix and tonsil, general effects are produced.
- Cross infection : When a patient is suffering from a disease and new infection is set up from another host or external source.
- Nosocomial infection : Cross infection occurring in hospital.
- Subclinical infection : It is one where clinical affects are not apparent.

### **Causative agents of infections**

- Parasites : They are organisms that can establish themselves and multiply in hosts. They may be pathogens or commensal.
- Pathogens : Organisms that are capable of producing disease in a host. On the contrary commensal microbes can live in a host without causing any disease.
- Man : Man is himself a common source of infection from a patient or carrier.  
Carrier is a person harboring pathogenic organism without causing any disease to him.
- Animals : Infectious diseases transmitted from animals to man is called zoonosis.
- Insects : The diseases caused by insects are called arthropod borne disease.
- Soil : Spores of tetanus bacilli, Gas-gangrene infection remain viable in soil for a long time.
- Water : Vibrio cholera, infective hepatitis virus (Hepatitis A and Hepatitis E) may be found in water.
- Food : Contaminated food may be source of infection. Presence of pathogens in food may be due to external contamination, (e.g. food poisoning by Staphylococcus
- Sexual Contact : syphilis, gonorrhea.

### **Methods of spread of infection**

- Inhalation : influenza, tuberculosis, small pox, measles, mumps, etc.
- Ingestion : cholera (water), food poisoning (food) and dysentery (hand borne).

Inoculation	: tetanus (infection), rabies (dog), arbovirus (insect) and serum hepatitis
Congenital	: syphilis, rubella, toxoplasmosis, cytomegaloviruses
Insects	: Insects like mosquitoes, fleas, lice that transmit infection are called vector. Transmission may be mechanical (transmission of dysentery or typhoid bacilli by housefly) and they are called mechanical vector. They are called biological vector if pathogen multiplies in the body of vector, e.g. Anopheles mosquito in Malaria. Some vectors may act as reservoir host, (e.g. ticks in Relapsing fever and Spotted fever).
Iatrogenic	: infection induced by physician
Nosocomial	: cross infections obtained in the hospitals

Pathogenicity is referred to the ability of microbial species to produce disease.

Virulence is referred to the ability of microbial strains to produce disease.

Endemic diseases are ones that are constantly present in a particular area. Typhoid fever is endemic in most parts of India.

Epidemic disease is one that spreads rapidly, involving many persons in an area at the same time. Influenza causes annual winter epidemics in the cold countries.

Pandemic is an epidemic that spreads through many areas of the world involving very large numbers of persons within a short period (Influenza, cholera, plague).

### Historical aspects

History of microbiology is the history of medical science too. The concept of "Spontaneous generation", made Aristotle and others to believe that living organisms could develop from non-living materials. Antony Van Leeuwenhoek, with the help of his microscope described Protozoa, basic types of bacteria, yeasts and algae as "Animalcules". Louis Pasteur is the father of Medical Microbiology. He invented the processes of pasteurization, fermentation and developed effective vaccines for (rabies and anthrax) diseases. He coined the term "microbiology", aerobic, anaerobic etc. He disproved the theory of spontaneous germination and demonstrated that anthrax was caused by bacteria and produced a vaccine for the disease too.

Joseph Lister concluded that wound infections were due to microorganisms. He also devised a method to destroy microorganisms in the operation theatre by using carbolic acid. Robert Koch demonstrated the role of bacteria in causing disease.

He perfected the technique of isolating bacteria in pure culture. Koch's postulates were much useful in differentiating pathogens from normal flora

Edward Jenner discovered the technique of vaccination which was helpful in preventing small pox. Alexander Fleming discovered antibiotic penicillin from penicillium notatum that destroy several pathogenic bacteria. Paul Ehrlich discovered the treatment of syphilis by using arsenic He studied toxins and antitoxins and laid foundation of biological standardization. Dmitri Ivanowski published the first evidence of a pathogenic agent-virus from tobacco mosaic disease, effectively launching the field of virology.

#### 4.1.2 STRUCTURE AND CLASSIFICATION OF BACTERIA

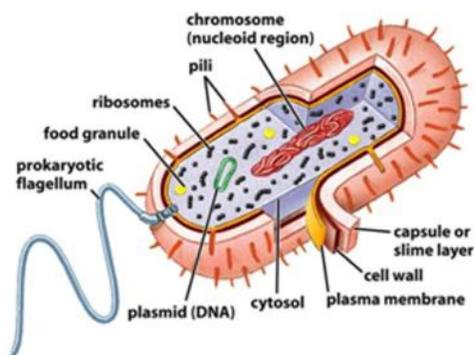
##### Structure

Based on the composition of their cells, all living organisms under the kingdom Protista are broadly classified into eukaryotes. In prokaryotes the cells having simple genetic structures, enzymes and divided by binary fission and not through mitosis. No nucleus or membrane bound organelles are present. Bacteria are included among prokaryotes. Eukaryotes like protozoa or fungi, cellular organisation is complex, and divide by mitosis.

Bacteria consists of a single cell with a simple internal structure. It has single loop of DNA and in a twisted thread-like mass called the nucleoid. Some bacteria have an extra circle of genetic material called plasmid that makes the bacterium resistant to a certain antibiotic. Typically a bacteria have few micrometres in length and can be observed by microscope. (One micron means 1/1000 of a mm)

Bacterial cells are generally surrounded by two protective coverings: an outer cell wall and an inner cell membrane. Some bacteria may even have a third, outermost, protective layer called the capsule. The cell wall contains peptidoglycan and other components that contribute to their pathogenicity. The cell wall protects a cell from toxic substances and is the site of action of several antibiotics. The plasma membrane encloses the cytoplasm.

Bacterial capsule is a well-organized layer that is not easily washed off. Slime is an unorganized material that is removed easily present in few bacteria over the capsule. Capsules and slime



layers are usually composed of polysaccharides. Capsules give protection to bacteria and is antigenic, having much diagnostic importance.

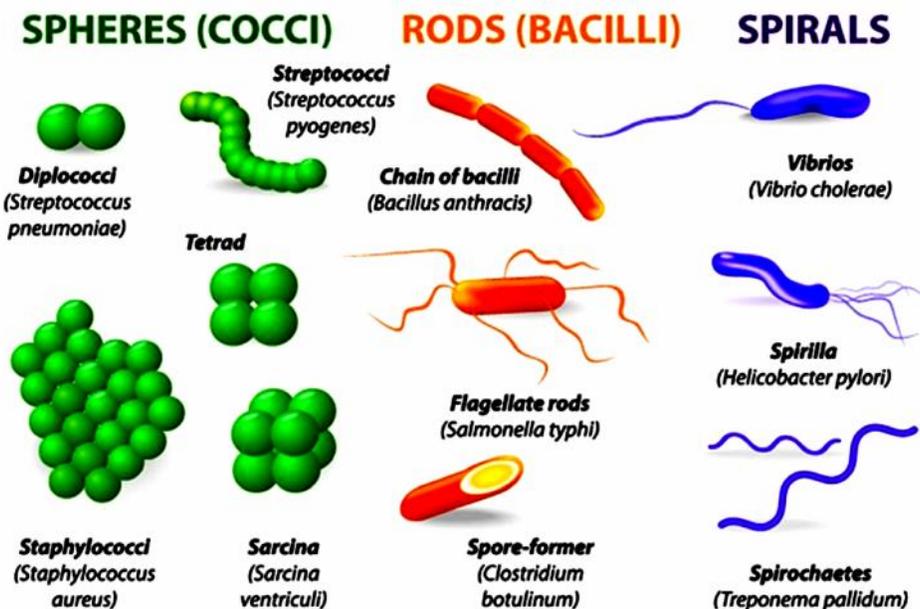
Bacterial surfaces may have extensions called flagella or pili. Flagella are the organ for locomotion and the function of pili or fimbria is adhesion or attachment. Most motile bacteria move by flagella. The detailed structure of a flagellum can only be seen in the electron microscope. Bacterial species often differ in their flagella distribution. Many gram-negative bacteria have short, fine, hair-like appendages thinner than flagella called fimbriae which are not involved in motility.

Bacteria lack membrane-bound organelles. However, like eukaryotic cells bacteria also contain ribosomes. They are spherical and are the centres of protein synthesis.

Many gram-positive bacteria can form a special resistant, dormant structure called endospore. Endospores develop within vegetative bacterial cells of several genera like Bacillus and Clostridium. These are resistant to heat, ultraviolet radiation, gamma radiation, chemical disinfectants, and desiccation. Because of their resistance, several species of endospore-forming bacteria are dangerous pathogens like Clostridium tetani, Clostridium botulinum etc.

A variety of inclusion bodies, granules of organic or inorganic material is present in the cytoplasmic matrix. These bodies are used for storage. Many bacteria store phosphate as polyphosphate granules or volutin granule.

### Classification of Bacteria



Based on Morphology bacteria can be classified into six major groups

**1. Cocci** - These are spherical or oval cells. On the basis of arrangement of individual organisms they can be described as

- **Monococci** (Cocci in singles) - Monococcus spp.
- **Diplococci** (Cocci in pairs) - Streptococcus pneumoniae
- **Staphylococci** (Cocci in grape-like clusters) - Staphylococcus aureus
- **Streptococci** (Cocci in chains) - Streptococcus pyogenes
- **Tetrad** (Cocci in group of four) - Micrococcus spp.
- **Sarcina** (Cocci in group of eight)

**2. Bacilli** - These are rod-shaped bacteria e.g. E.coli, Klebsiella etc.

Chinese-letter pattern : Corynebacterium diphtheriae

Cocci bacilli : Proteus

**3. Vibrio**-coma shaped e.g. : V.cholerae

**4. Actinomycetes**-These are rigid organisms like true bacteria but they resemble fungi in that they exhibit branching and tend to form filaments. E.g. A.israeli

**5. Spirochaetes**-These are relatively longer, slender, non-branched microorganisms of spiral shape having several coils. E.g. Leptospira, Treponema etc.

**6. Mycoplasmas**-They occur in round or oval bodies and in interlacing filaments. These bacteria lack in rigid cell wall (cell wall lacking) and are highly pleomorphic and of indefinite shape. E.g. M. pneumoniae

**Based on Oxygen dependence bacteria are classified into different groups**

**Aerobes** are those bacteria that require oxygen for growth.

**Obligate aerobe** those bacteria that cannot grow without oxygen for growth

e.g. Mycobacterium tuberculosis and Vibrio cholerae

**Obligate Anaerobes** grow in absence of free oxygen, e.g. Clostridium, Bacteroides. They will die on exposure to even trace amounts of oxygen.

**Facultative anaerobes** are usually aerobes and grow under both aerobic and anaerobic conditions.

Most bacteria of medical importance are facultative anaerobes, e.g. Enterobacteriaceae.

**Microaerophils** grow best in oxygen less than that present in the air, e.g. Campylobacter. Bacteria that grow well with CO<sub>2</sub> rich environment is called carboxyphilic or capnophilic. Eg Brucella abortus.

**Based on Motility bacteria are classified into motile and non motile**

Motile Bacteria eg:- ( E.coli,vibrio etc.)

Non-motile eg:- (Klebsiella, staphylococci)

**4.1.3 Sterilization and disinfection**

**Sterilization** is the removal of all kinds of microbes (bacteria, mycobacteria, viruses, & fungi) in a material or on the surface of an object both from vegetative and spore state. Sterilization can be achieved by physical and chemical means

**Disinfection** is the process of elimination of most pathogenic microorganisms (excluding bacterial spores) on inanimate objects. Disinfection can be achieved by physical or chemical methods.

**Physical methods of sterilization**

They are,

**Sunlight:** The microbicidal activity of sunlight is mainly due to the presence of ultra violet rays in it.

**Heat:** Heat is considered to be most reliable method of sterilization of articles that can withstand heat. Those articles that cannot withstand high temperatures can still be sterilized at lower temperature by prolonging the duration of exposure. Moist heat is superior to dry heat in action. Temperature required to kill microbe by dry heat is more than the moist heat.

**Dry heat:** acts by oxidative effects as well as denaturation and coagulation of proteins and toxic effects of elevated levels of electrolytes.

**Red heat:** Articles such as bacteriological loops, straight wires, tips of forceps are sterilized by holding them in Bunsen flame till they become red hot.

**Flaming:** This is a method of passing the article over a Bunsen flame, but not heating it to redness. Articles such as scalpels, mouth of test tubes, flasks, glass slides and cover slips are passed through the flame a few times.

**Incineration:** This is a method of destroying contaminated material by burning them in incinerator. Articles such as soiled dressings, animal carcasses, pathological material and bedding etc. should be subjected to incineration. The limitation of this method is that articles can't be reused after incineration

**Hot air oven:** This method was introduced by Louis Pasteur. Articles to be sterilized are exposed to high temperature (160°C) for duration of one hour in an electrically heated oven. Since air is poor conductor of heat, even distribution of heat throughout

the chamber is achieved by a fan. The heat is transferred to the article by radiation, conduction and convection. The oven should be fitted with a thermostat control, temperature indicator, meshed shelves and must have adequate insulation. Metallic instruments (like forceps, scalpels, and scissors), glassware, swabs, oils, grease, petroleum jelly and some pharmaceutical products are sterilized by this method.

### **Sterilization process by hot air oven**

Articles to be sterilized must be perfectly dry before placing them inside hot air oven. Articles must be placed at sufficient distance so as to allow free circulation of air in between. Mouths of flasks, test tubes and both ends of pipettes must be plugged with cotton wool. Articles such as petri dishes and pipettes may be arranged inside metal canisters and then placed. Individual glass articles must be wrapped in kraft paper or aluminum foils. Holding time is 160°C for one hour



The hot air oven must not be opened until the temperature inside has fallen below 60°C to prevent breakage of glassware

A biological method is used as a control of sterilization.  $10^6$  spores of *Bacillus subtilis* or *Clostridium tetani* on paper strips are placed inside envelopes and then placed inside the hot air oven. Upon completion of sterilization cycle, the strips are removed and inoculated into thioglycollate broth or cooked meat medium and incubated at 37°C for 3-5 days. Proper sterilization should kill the spores and there should not be any growth. The Advantages of hot air oven is that it is an effective method of sterilization of heat stable articles. The articles remain dry after sterilization. This is the only method for sterilizing oils and powders.

The disadvantages are hot air has poor penetration, Cotton wool and paper may get slightly charred, glasses may become smoky and takes longer time compared to autoclave. Culture media cannot be sterilized by this method.

**Moist heat** acts by coagulation and denaturation of proteins.

### **Temperature below 100°C**

**Pasteurization:** This process was originally employed by Louis Pasteur. Currently this procedure is employed in dairy industry.

**Vaccine bath/ Serum bath:** The contaminating bacteria in a vaccine preparation can be inactivated by heating in a water bath at 60°C for one hour. The contaminating bacteria in a serum preparation can be inactivated by heating in a serum bath at 56°C for one hour on several successive days. Only vegetative bacteria are killed and spores survive.

### Temperature at 100°C

**Inspissation:** This is a technique to solidify as well as disinfect egg and serum containing media. The medium containing serum or egg are placed in the slopes of an inspissator and heated at 80-85° C for 30 minutes on three consecutive days.

**Boiling:** Boiling water (100°C) kills most vegetative bacteria and viruses immediately. Some bacterial spores are resistant to boiling and survive; hence this is not a substitute for sterilization. When absolute sterility is not required, certain metal articles can be disinfected by placing them in boiling water for 10-20 minutes. The lid of the boiler must not be opened during the period.

**Steam at 100°C:** Instead of keeping the articles in boiling water, they are subjected to free steam at 100°C. Traditionally Arnold's and Koch's steamers were used. An autoclave (with discharge tap open) can also serve the same purpose. A steamer is a metal cabinet with perforated trays to hold the articles and a conical lid. The bottom of steamer is filled with water and heated. The steam that is generated sterilizes the articles when exposed for a period of 90 minutes.

**Tyndallisation** -The process that is known as tyndallisation or intermittent sterilization is employed by steamer (at 100°C) Sugar and gelatin in medium may get decomposed on autoclaving, hence they are exposed to free steaming for 20 minutes for three successive days. The vegetative bacteria are killed in the first exposure and the spores that germinate by next day are killed in the subsequent days. The success of the process depends on the germination of spores.

### Temperature above 100°C

**Autoclave:** Sterilization can be effectively achieved at a temperature above 100°C using an autoclave. Water boils at 100°C at atmospheric pressure, but if pressure is raised, the temperature at which the water boils also increases. In an autoclave the water is boiled in a closed chamber. As the pressure rises, the boiling point of water also rises. At a pressure of 15 lbs inside the autoclave, the temperature is said to be 121°C. Exposure of articles to this temperature for 15 minutes sterilizes them. Advantages of steam over dry air is that it has more penetrative power. Condensation of steam on cooler surface releases latent heat, and condensation of steam draws in fresh steam.

**Operation of Autoclave:** A simple autoclave has vertical or horizontal cylindrical body with a heating element, a perforated tray to keep the articles, a lid that can be fastened by screw clamps, a pressure gauge, a safety valve and a discharge tap. The articles to be sterilized must not be tightly packed. The screw caps and cotton plugs must be loosely fitted. The lid is closed but the discharge tap is kept open and the water is heated. As the water starts boiling, the steam drives air out of the discharge tap. When all the air is displaced and steam start appearing through the discharge tap, the tap is closed. The pressure inside is allowed to rise upto 15 lbs per square inch. At this pressure the articles are held for 15 minutes, after which the heating is stopped and the autoclave is allowed to cool. Once the pressure gauge shows the pressure equal to atmospheric pressure, the discharge tap is opened to let the air in. The lid is then opened and articles removed.



Articles sterilized by autoclaving are Culture media, dressings, linen etc. Autoclaving is the very effective method of sterilization, quicker than hot air oven. Drenching and wetting of articles, trapped air may reduce the efficacy, takes long time to cool are the disadvantages of the method.

Biological method of sterilization control (paper strip containing  $10^6$  spores of bacillus stearothermophilus) is used in Autoclaves

**RADIATION**-Two types of radiation are used, ionizing and non-ionizing.

Non-ionizing rays are low energy rays with poor penetrative power (eg. UV rays and Infra-red rays). UV rays are used for disinfecting laboratories, safety hood etc. Ionizing rays are high-energy rays with good penetrative power. (eg. Gamma rays and X rays) Gamma radiations are used to sterilize syringes and catheters

**FILTRATION:** It is a method used to remove bacteria from heat labile liquids such as serum, antibiotic solutions, sugar solutions, urea solution etc. It is achieved by using different type of filters like candle filters, Membrane filters Asbestos filters and Sintered glass filters Membrane filters are commonly used to remove particles from solutions that can't be autoclaved.

**SONIC AND ULTRASONIC VIBRATIONS:** Sound waves of frequency  $>20,000$  cycle/second kills bacteria and some viruses on exposing for one hour.

**CHEMICAL METHODS OF DISINFECTION:** Disinfectants are those chemicals that destroy pathogenic bacteria from inanimate surfaces. Different disinfectants have different target ranges. Some chemical have very narrow spectrum of activity and some have very wide. Those chemicals that can be safely applied over skin and mucus membranes are called antiseptics. Bacteriostatic chemicals are those inhibit multiplication of bacteria without killing them. Bactericidal is that chemical that can kill or inactivate bacteria. Mode of action of various Chemical Disinfectants include. Protein coagulation, disruption of cell membrane, enzyme damage etc

**Different chemicals used as disinfectants includes**

**ALCOHOLS:** Eg: Ethyl alcohol, isopropyl alcohol and methyl alcohol. 70% ethyl alcohol (spirit) is used as antiseptic on skin.

**ALDEHYDES** Eg: Formaldehyde, and Gluteraldehyde

40% Formaldehyde (formalin) is used for surface disinfection and fumigation operation theatres, biological safety cabinets, wards, etc.

**PHENOL** . Examples: 5% phenol, Cresol, Lysol, chlorhexidine, Dettol

**HALOGENS:**. Examples: Chlorine compounds (chlorine, bleach, hypochlorite) and iodine compounds Tincture of iodine (2% iodine in 70% alcohol) chlorine is used for disinfecting water supplies

**HEAVY METALS** : Eg. Mercuric chloride, silver nitrate, copper sulfate,

**SURFACE ACTIVE AGENTS** : Examples: These are soaps or detergents.

**DYES:** Eg: Aniline dyes such as crystal violet, malachite green and brilliant green

**ETHYLENE OXIDE (EO):** It is an alkylating agent capable of killing spores rapidly.

**BETA-PROPIOLACTONE (BPL)** : It is an effective sporicidal alkylating agent and is a carcinogen.

## Unit 4.1.4 Microbiology

### Bacterial growth

Bacteria divide by binary fission. Bacterial cell reaches a certain size and divide to form two daughter cells .The time taken between two cell divisions under optimal conditions is called generation time. Generally it is 20 minutes for most of the bacteria. But for Mycobacterium tuberculosis, the bacilli that cause TB it is about 20 Hr. s and for lepra bacilli that cause leprosy (M.leprae ) it is nearly 20 days in culture.

The essential growth requirements for a bacterial cell are water, carbon, protein, nitrogen, inorganic salts particularly sodium, potassium and trace elements such as magnesium, iron, manganese, vitamins, amino acids, purines and pyrimidine etc. All the above growth requirements may be present in a natural environment, but should be provided in required amounts when an invitro cultivation is sought. Culture media components are available commercially in separate or as a single dehydrated (Powder) form. Environmental Factors affecting growth like oxygen, carbon dioxide, temperature, light moisture, hydrogen ion concentration etc; are equally important in producing bacterial growth.

Most of the bacteria are aerobes which require oxygen for the growth, anaerobic bacteria grow well in the absence of oxygen. Bacteria that grow well with CO<sup>2</sup> rich environment is called carboxyphilic or capnophilic. Eg. *Brucella abortus*.

Temperature at which bacteria grow best is known as optimum temperature. Optimum temperature for growth of common pathogenic bacteria is 37°C. Bacteria grow well at 30-37°C. are mesophilic. Bacteria may be psychrophilic (below 20°C) or thermophilic (55-80 °C) too. Increased temperature may kill bacteria.

pH of the medium is another important factor. Optimum pH of the Most pathogenic bacteria is slightly alkaline.

### **Culture media**

Culture media is the substance that supports bacterial growth in vitro. It is prepared by adding necessary chemicals and growth factors in exact composition same as that in a natural environment. Cultivating bacteria as pure culture and isolated colonies are necessary for the identification of bacteria from a mixed population.

**Composition of culture media:** An artificial culture medium must provide all the nutritional components that a bacterium gets in its natural habitat. Most often, a culture medium contains water, a source of carbon & energy, source of nitrogen, trace elements and some growth factors. Besides these, the pH of the medium must be set accordingly. Some of the ingredients of culture media include water, agar, peptone, casein hydrolysate, meat extract, yeast extract and malt extract.

### **Classification of culture media**

#### **Based On Consistency**

**Liquid media:** Liquid media are otherwise referred as "broths" (e.g. nutrient broth). In liquid medium, bacteria grow uniformly producing general turbidity. Properties of bacteria are not visible in liquid media and presence of more than one type of bacteria cannot be detected. These are available for use in test-tubes, bottles or flasks

**Solid media:** Any liquid medium can be rendered by the addition of certain solidifying agents. Agar agar (simply called agar) is the most commonly used solidifying agent. It is a non-nutritious polysaccharide obtained from the cell membranes of some species of red algae. It melts at 95° C and solidifies at 42° C. It is not hydrolyzed by most bacteria and is usually free from growth promoting or growth retarding substances. Agar is available as fibers (shreds) or as powders.

**Semi-solid agar:** Reducing the amount of agar to 0.2-0.5% renders a medium semi-solid. Such media are fairly soft and are useful in demonstrating bacterial motility (mannitol motility medium). Certain transport media such as Stuart's and Amies media are semi-solid in consistency.

**Biphasic media:** Sometimes, a culture system comprises of both liquid and solid medium in the same bottle. This is known as biphasic medium (Castaneda system for blood culture).

### **Based on nutritional component**

Most of the nutritional components are constant across various media, some bacteria need extra nutrients. Those bacteria that are able to grow with minimal requirements are said to be non-fastidious and those that require extra nutrients are said to be fastidious.

**Simple/Basal medium.** They basically support most non-fastidious bacteria. Peptone water, nutrient broth and nutrient agar are considered basal media.

**Complex medium :** Media other than basal media. They have added ingredients. They provide special nutrients.

**Synthetic or defined medium-**Media prepared from pure chemical substances and its exact composition is known (Simple media such as peptone water, nutrient agar can support most non-fastidious bacteria. Complex media such as blood agar have ingredients whose exact components are difficult to estimate. )

Special media are prepared for those organisms that need special nutritional requirements.

- Enriched media
- Enrichment media
- Selective media
- Indicator media
- Differential media
- Sugar media
- Transport media
- Media for biochemical reactions

**Enriched media:** Addition of extra nutrients in the form of blood, serum, egg yolk etc, to basal medium makes them enriched media. Enriched media are used to grow nutritionally exacting (fastidious) bacteria. Blood agar, chocolate agar, Loeffler's serum slope are few enriched media.

**Selective media:** The media are designed to inhibit unwanted commensal or contaminating bacteria and help to recover pathogen from a mixture of bacteria.. Any agar media can be made selective by addition of certain inhibitory agents that don't affect the pathogen. Various approaches to make a medium selective include addition of antibiotics, dyes, chemicals, alteration of pH or a combination of these.

E.g. : MacConkey's medium - gram negative bacteria  
 TCBS - *V.cholerae*  
 LJ medium - *M.tuberculosis*  
 Wilson and Blair medium - *S.typhi*  
 Potassium tellurite medium - *Diphtheria bacilli*

**Enrichment media** are liquid media that also serves to inhibit commensals in the clinical specimen. Selenite F broth, tetrathionate broth and alkaline peptone water are used to recover pathogens from faecal specimens.

E.g. : Selenite F Broth - for the isolation of *Salmonella*, *Shigella*  
 Alkaline Peptone Water - for *Vibrio cholera*

**Differential media or indicator media** are designed in such a way that different bacteria can be recognized on the basis of their colony colour. Various approaches include incorporation of dyes, metabolic substrates etc, so that those bacteria that utilize them appear as differently coloured colonies. Such media are called differential media or indicator media. Examples: MacConkey's agar, CLED agar, TCBS agar, XLD agar etc.

**Transport media:** Clinical specimens must be transported to the laboratory immediately after collection to prevent over growth of contaminating organisms or commensals. This can be achieved by using transport media. VR Fluid, Stuart's medium, Amie's medium are examples for transport media.

**Based on Oxygen requirement** media can be of two types Aerobic media & Anaerobic media

**Anaerobic media :** Anaerobic bacteria need special media for growth because they need low oxygen content, reduced oxidation -reduction potential and extra nutrients. Eg: Robertson's cooked meat medium, Thioglycolate medium.

### Preparation of Common media

**Peptone Water** It contains 1% peptone and 0.5% NaCl in distilled water. pH is adjusted to 7.4 -7.6 It is used for preparation of sugar media and alkaline peptone water.

**Nutrient Agar (NA)** : contains 0.5% peptone, 0.3% beef extract, and 1.5% agar pH is adjusted to 7.4 -7.6. The medium will support the growth of many organism which are not nutritionally fastidious (e.g. staphylococci, and enteric bacteria)

**Blood agar (BA)** Mammalian blood (usually sheep or horse), at a concentration of 5-10% is added to NA at a temperature about 50°C. BA are enriched, differential media used to isolate fastidious organisms and detect hemolytic activity.

**Chocolate agar (CA)** is a non-selective, enriched medium containing red blood cells that have been lysed by slowly heating to 80°C. It is prepared by heating blood Agar until medium becomes brown or chocolate in color. Chocolate agar is used for growing fastidious bacteria, such as Haemophilus influenzae. On heating blood releases both X and V growth factors.

**MacConkey Agar (MA)** is designed to grow gram-negative bacteria and differentiate them as lactose fermenters (LF) and non-lactose fermenters (NLF). It contains Peptone, bile salts (to inhibit most gram-positive bacteria), Lactose, Neutral red as indicator and agar.

**Bacteriological wire loop** : It is a simple tool used for introducing microorganism in to a culture media (inoculation). The loop of wire is made of platinum, tungsten or chrome. The small loop at the end has a diameter of about 5 mm. Straight wire is used for stab culture The inoculation loop is sterilized by red heat.

### Inoculation of culture media

Bacterial culture are usually done

- To isolate bacteria in pure cultures from clinical specimens.
- To demonstrate their properties.
- To obtain sufficient growth for the preparation of antigens and for other tests.
- To determine sensitivity to antibiotics.
- To estimate viable counts.
- Maintain stock cultures.

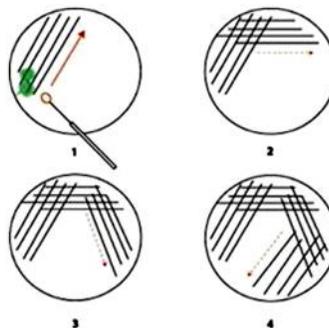
**Inoculation methods** include:

- Streak culture

- Lawn culture
- Stroke culture
- Stab culture
- Pour plate method
- Liquid culture
- Anaerobic culture methods

### Streak culture

- Used for the isolation of bacteria in pure culture from clinical specimens.
- Platinum wire or Nichrome wire is used.
- One loopful of the specimen is transferred onto the surface of a well dried plate.
- Spread over a small area at the periphery.
- The inoculum is then distributed thinly over the plate by streaking it with a loop in a series of parallel lines in different segments of the plate.
- On incubation, separated colonies are obtained over the last series of streaks



### Lawn Culture

- Provides a uniform surface growth of the bacterium.

Uses are

- For bacteriophage typing.
- Antibiotic sensitivity testing.
- In the preparation of bacterial antigens and vaccines.

Lawn cultures are prepared by flooding the surface of the plate with a liquid suspension of the bacterium or swabbing on the surface of the plate.

### Stroke Culture

Stroke culture is made in tubes containing agar slope / slant It is used to provide a pure growth of bacterium for slide agglutination and other diagnostic tests and certain biochemical tests Eg: TSI, Citrate utilization.

### Stab culture

Prepared by puncturing a suitable medium - gelatin or glucose agar with a long, straight, charged wire.

Uses of stab culture are

- Motility detection in semisolid media.

- Demonstration of gelatin liquefaction.

### **Pour plate culture**

- Agar medium is melted (15 ml) and cooled to 45°C.
- 1 ml of the inoculum is added to the molten agar.
- Mix well and pour to a sterile petri dish. Allow it to set.
- Incubate at 37°C, colonies will be distributed throughout the depth of the medium.

Uses are

- Gives an estimate of the viable bacterial count in a suspension.
- For the quantitative urine cultures.

### **Liquid cultures**

- Liquid cultures are inoculated by touching with a charged loop or by adding the inoculum with pipettes or syringes.
- Used for obtaining uniform growth of bacteria for motility testing and blood culture etc.

### **Anaerobic culture**

Anaerobic condition is created by production of vacuum (Incubate the cultures in a vacuum desiccator), displacement of oxygen with other gas (Displacement of oxygen with hydrogen, nitrogen, helium or CO<sup>2</sup>) and by absorption of oxygen ( gas pack). Different methods are available

- Chemical method--Alkaline pyrogallol absorbs oxygen.
- McIntosh - Fildes' anaerobic jar
- Gaspak

Commercially available disposable envelope. Gas-pak contains pellets of sodium borohydride, cobalt chloride, citric acid and sodium bicarbonate.

These chemicals generate hydrogen and carbon dioxide when water is added (about 10 ml) (some gaspak do not need water). After the inoculated plates are placed inside an air-tight jar, the packet of "Gas-pak" with water added is kept inside and the lid is tightly closed. Indicator is used is reduced methylene blue. It is colourless anaerobically and Blue in colour on exposure to oxygen

## Unit 4.1.5 Basic Identification Techniques

### Introduction

The main function of all diagnostic bacteriology laboratories is the detection and identification of microorganisms in a variety of samples. Additionally, Antibiotic sensitivity testing of the isolates is of major importance. Now days conventional Identification techniques mostly has been replaced by rapid techniques. But still staining techniques microscopy remain as the gold standard methods for identification of bacteria. Supplementation of biochemical investigations mostly detecting bacterial enzymes or metabolic end products confirms the diagnosis in a species level.

There are several methods to identify the different type of bacteria.

1. Isolation in pure form
2. Staining reaction
3. Morphology of bacterial colony
4. Cultural characteristics
5. Metabolism
6. Biochemical properties

### Detection of Bacterial Motility

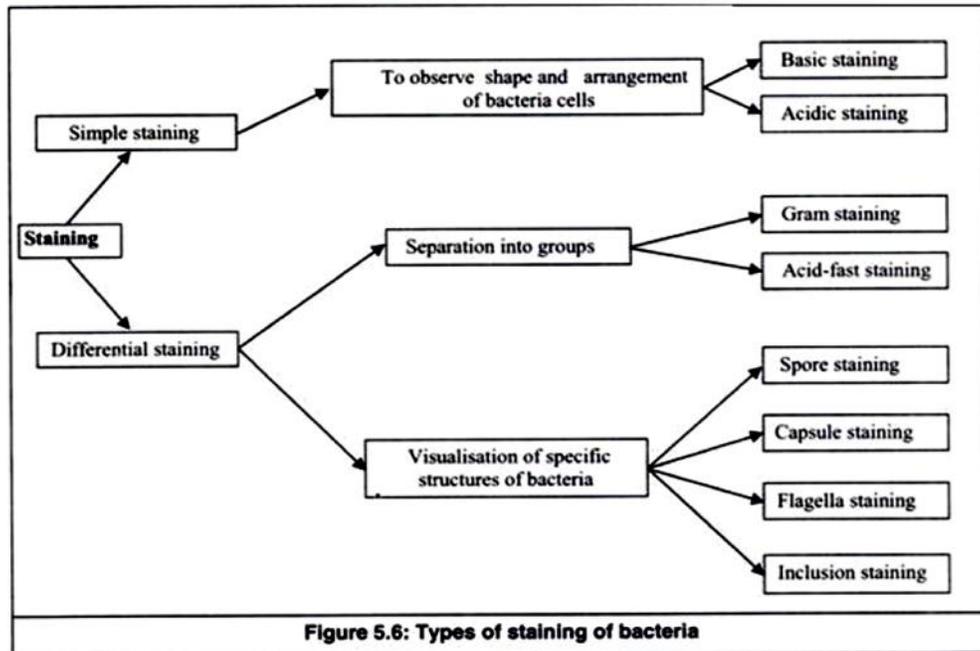
Bacterial Motility may be studied by a variety of techniques. The movement of living bacteria may be examined in solid or semisolid agar media, microscopically in wet, unstained preparations or in stained preparations. There are different methods for motility detection.

#### Hanging drop method

In this method a drop of culture is placed on a coverslip that is encircled with petroleum jelly (or any other sticky material). The coverslip and drop are then inverted over the well of a depression slide and examine under microscope for bacterial movement. The drop hangs from the coverslip, and the petroleum jelly forms a seal that prevents evaporation. It is the quickest means for determining motility.

#### Staining reaction

The refractive index of bacterial cells is very close to that of water and to that of the glass slide, Therefore, it is very difficult to observe them clearly. To overcome this difficulty, the cells are coloured by stains, which impart deep colour to the cells and light colour to the surrounding medium, in which they are suspended.



Staining of bacteria is of different types as described below

### Simple Staining

Here, only one stain is used. E.g. Methylene Blue, Basic fuchsin etc. This staining is used to observe shape (cocci, bacilli, vibrio, spirilli) and arrangement (single, pair, tetrad, chain, cluster) of bacteria.

### Differential Staining:

Differential staining methods are performed to differentiate bacteria into different groups based on their staining characteristics. Gram staining and Acid Fast staining are differential staining techniques

Gram Staining is performed to differentiate gram-positive and gram-negative bacteria. It is based on the chemical composition of their cell wall. Gram staining uses crystal violet as primary stain, iodine as a mordant, and dilute carbol fuchsin or safranin as counterstain. The cell wall of gram-positive bacteria is rich with peptidoglycan and teichoic acid which are present in small amounts in gram-negative bacteria. Major content of the cell wall of a gram-negative bacteria is lipopolysaccharide. In spite of decolourisation gram-positive bacteria retains the primary stain iodine complex and remains purple. But gram-negative bacteria undergo decolourisation and takes the colour of the counter stain and appear as red. Any mechanical damage to the cell wall cause gram variability in staining.

In Gram staining Crystal violet is used as primary stain, Grams Iodine as the mordant, Ethyl alcohol / acetone as the decolouriser and Safranin/dilute Carbol fuchsin/Neutral red as the counter stain.

Acid-fast Staining (Ziehl-Neelsen staining) is performed to differentiate between acid-fast and non-acid-fast bacteria and is used to stain Mycobacterium tuberculosis that do not take colour during staining procedures like Gram staining. The stains used are the red colored Carbol fuchsin that stains the bacteria and a counter stain like Methylene blue or Malachite green. Primary stain binds cell wall mycolic acids. Intense decolorization does not release primary stain from the cell wall of Acid fast bacillus. Colour of AFB-based on primary stain used. Counterstain provides contrasting background.

### **Biochemical Tests**

Different tests based on the biochemical property of microorganism helps in the identification and confirmation of different bacteria.

Catalase Test demonstrates the presence of enzyme catalase in the organism. Staphylococcus gives Positive catalase, Streptococcus gives Negative catalase results.

Coagulase Test is used to differentiate Staphylococcus aureus (positive) from Staphylococci epidermidis which is coagulase negative.

Oxidase test is used to determine the presence of bacterial cytochrome oxidase enzyme. Pseudomonas species gives positive reaction and E coli gives negative results.

IMViC tests are used to differentiate the members of Enterobacteriaceae. These are the Indole test, the Methyl Red, Voges-Proskauer tests and the Citrate test.

## **4.1.6 Immunology and its diagnostic applications**

### **Introduction**

The body is protected from infectious agents and other harmful substances by a variety of cells and molecules that make up the immune system. Immunity is the ability of the human body to tolerate the presence of material indigenous to the body (self), and to eliminate foreign (non-self) material. Foreign substances such as viruses, bacteria, toxins, and parasites are surrounded by antigens that, when introduced into the body, are capable of inducing a response by the immune system. This discriminatory ability provides protection from infectious disease, since most agents or associated toxins are identified as foreign by the immune system. The immune system - includes all parts of the body that help in the recognition and destruction of

foreign materials. White blood cells, phagocytes and lymphocytes, bone marrow, lymph nodes, tonsils, thymus spleen are all part of the immune system. The study of immunity and various immunological reactions are called Immunology

### Types of immunity

**Passive Immunity:** Antibodies from another person or animal that can be injected or transfused. Called passive because the individual did not create the antibodies, but instead received pre-formed antibodies. Protection is effective, but duration is short lived and no memory is created. Examples of passive immunity are maternal antibodies (trans-placental and breast milk) and injected antibodies (e.g., rabies, varicella, and tetanus immune globulins).

**Active Immunity:** When the body is exposed to a foreign substance the cells of the immune system "actively" respond. Active immunity is further divided into categories:

Innate Immunity - protective mechanisms we are born with.

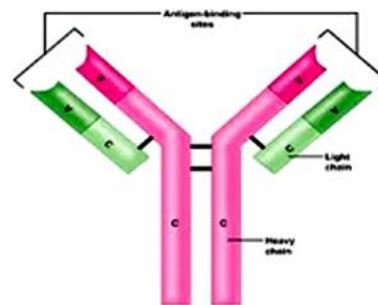
Adaptive Immunity - cell mediated immunity and humoral immunity

### Antigen

Antigen is usually a foreign molecule that triggers an immune response when introduced into the body.

### Antibody

Antibody is chemically globulin molecule secreted in response to a specific antigen. Each antibody consists of four polypeptides- two heavy chains and two light chains joined to form a "Y" shaped molecule. light chains may be either kappa or Lambda. the heavy chain may be Alpha, beta, mu,delta and epsilon. According to the type of heavy chain present, the antibodies are classified into: IgM, IgG, IgA, IgD, and IgE



Structure of Antibody

**IgM:** A valuable diagnostic marker for infectious disease because it is usually the first immunoglobulin made following Antigen exposure

**IgG:** The most abundant class of antibody, constituting approximately 80% of all antibodies in serum. It is the only antibody that crosses the placenta.

### Antigen-Antibody Reactions

The antigens- antibody reactions forms the basis of antibody mediated immunity in infectious diseases, allergy etc. The specificity of antigen-antibody interactions has

led to the development of a variety of immunologic assays, which can be used to detect either the antibody or antigen that may aid in the diagnosis of infections. The antigen and antibody reactions are specific and the combination is firm but reversible it may be influenced by the affinity and avidity of the reaction.

Affinity refers to the intensity of attraction between the antigen and antibody molecule

Avidity is the strength of bond after the formation of antigen antibody complexes

## Serology

**Antigen** - antibody interactions in vitro are known as serological reactions. The important parameters of serological tests are

**Specificity** - is the ability of test to detect reactions between homologous antigens and antibodies only. False positive reactions are minimal in a highly specific test.

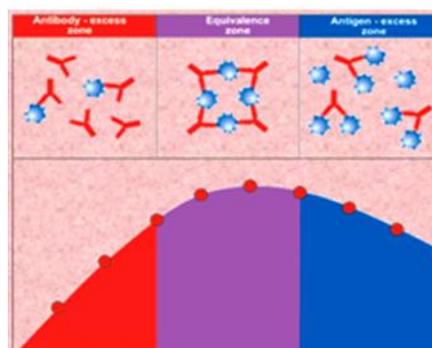
**Sensitivity** - refers to the ability of test to detect very minute quantities of antigen or antibody. In a highly sensitive test, false negative results are minimal.

Titer of the serum is the highest dilution of serum that shows an observable reaction with the antigen in the particular test. The titer is determined by serially diluting the serum fraction of blood and assaying (testing) each dilution of the serum with the antigen of interest. The last dilution of serum sample that responds in the assay determines the titer. The greater the concentration of the specific antibody in the serum sample, the higher the titer. A low or undetectable titer indicates very little antibody present in the serum.

## Prozone Phenomenon

The antigen-antibody reactions will be greatly influenced by the proportion of antigens and antibodies. The reaction will be more stronger if antigen and antibodies will be in a optimum concentration( Zone of equivalence), the excess amount of antibody (Prozone) often result in the false negative result i.e failer of reaction. Prozone effects can be seen in many types of immunoassays.

There are several antigen-antibody reactions used in vitro which includes Agglutination, Precipitation, Neutralization, Complement fixation, Fluorescent -antibody technique antibody, ELISA- Enzyme linked immunosorbent assay, Radio immunoassay and Immuno chromatographic techniques.



### Precipitation Reaction

When a soluble antigen combines with its antibody in the presence of electrolytes (NaCl) at a suitable temperature and pH, the antigen and antibody complex forms an insoluble precipitate.

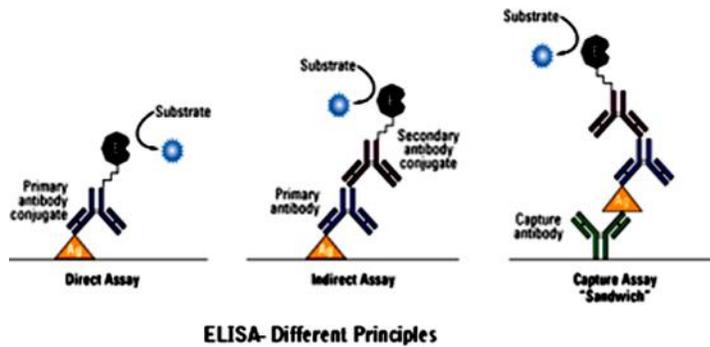
Instead of sedimenting at the bottom, precipitate of fine particles formed remains suspended as floccules and the reaction is known as flocculation. Examples are VDRL Test.

### Agglutination Reaction

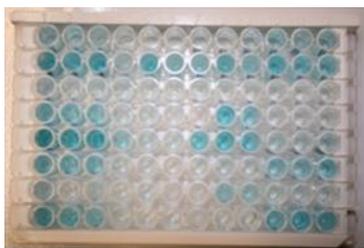
When a particulate antigen is mixed with its antibody in the presence of electrolytes, suitable temperature and pH produces clumps. Such reactions are called agglutination reaction. Agglutination reactions are more sensitive than precipitation reactions. Many soluble antigens can be made particulate by coating them onto RBC, or other particles such as latex or carbon and react with corresponding antibodies results in clumping called passive agglutination. Now a days many of the rapid diagnostic methods are using this principle for the diagnosis of diseases.

### Enzyme-Linked Immunosorbent Assay (ELISA)

This technique utilizes the specificity of antigen antibody reaction and sensitivity of enzyme. In this method, an enzyme labeled antigen or antibody is used to detect antigen or antibody from patient's sample. The result is obtained with the development of a colour formed as result of the action of enzyme on the substrate which is otherwise colourless. It is simple, Economical and more sensitive. ELISA is

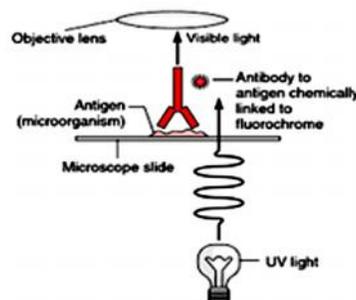


a quantitative method and available in different formats. Cassette ELISA is a simple modification. ELISA are now available for qualitative analysis. ELISA is widely used to detect various antigen and antibodies, in hormonal assays too.



## Immunofluorescence

In this method, antigen or antibody is labeled with fluorescent dyes such as fluorescein and rhodamine used in antigen-antibody reactions. The fluorescence produced is measured by fluorescent microscope. Immuno fluorescence method has wide diagnostic applications. In direct immune fluorescence method, unknown antigens are detected by fluorescent labeled antibodies. Indirect immune fluorescence method, antibodies in the patient's serum are detected.



**Immunofluorescence assay**

## Unit 4.1.7 Laboratory Diagnosis of Common Bacterial diseases

### Collection & transport of Specimens

Specimen collection forms the backbone of investigative procedures to isolate and identify causative agents in Microbiology. Specific procedures in collecting specimens will certainly improve the quality of services of Microbiology departments. Microbiological tests are expensive and technically demanding. Causal testing of Microbiological tests are counterproductive. The specimens collected and handled may directly affect the outcome of microbiological analysis, hence appropriate specimen management is critical to ensure laboratory effectiveness within an acceptable turn around time. Specific guidelines on specimen collection are necessary for optimal use of microbiology services.

Every laboratory should formulate guidelines on procedures for each major category of specimens.

### Collection

- Laboratory investigation should start immediately after collection of specimen
- Specimens obtained early, preferably prior to antimicrobial treatment
- Before doing anything, explain the procedure to patient and relatives
- When collecting the specimen, avoid contamination
- Take a sufficient quantity of material
- Follow the appropriate precautions for safety
- Containers must be leak proof, Unbreakable, for cultures containers must be sterile

### Labeling the sample

A properly labeled sample is essential to ensure that the results of the test match the

patient. The label must include Patient's name, Patient's hospital number, clinic number or ID number.

### **Specimen transport**

When facilities are not available to perform the desired tests at the place of collection or laboratory located far away, request the diagnostic laboratories to advice on transportation of specimens

Transport specimens to the laboratory as early as possible after collection. Specimens must be submitted for culture within 2 hours after collection, if delay is anticipated the specimens are to be preserved by refrigeration at 4°C except CSF specimen and cultured within 24 hours whenever possible.

Request forms for microbiological investigation should include name of patient, age, sex, IP/OP number, name of referring physician, type of specimen, Date of collection and investigations needed.

### **Specimen processing**

#### **Urine**

Collect mid-stream, clean catch specimen of Urine in a wide mouthed container from patients. A wet film is prepared under aseptic conditions to note the presence of pus cells and bacteria in the sample. Urine sample is then inoculated into Media like MA and BA and incubated at 37°C overnight.  $10^5$  CFU (colony forming unit) per ml of urine is considered as significant bacteriuria. Isolated colonies can be identified with biochemical tests. Expected pathogens include E.coli, Klebsiella, Pseudomonas, proteus etc. ABST should be performed on significant isolates.

#### **Blood**

Proper disinfection of the area is done with good antiseptic solution. The staff should be advised to disinfect the skin over vein, to use a fresh sterile syringe with sterile needle for the venipuncture before inoculating culture bottle. It is recommended that a minimum of 2 blood cultures from different sites should be submitted in order to acquire the optimal volume of blood (1 in 10 dilution) and to facilitate the interpretation of results. The sample is introduced in to sterile primary media like Bile Broth or Brain Heart Infusion Broth. Blood culture is requested when the patient is suspected of septicemia, Bacterimia, Subacute bacterial Endocarditis(SABE) and in some systemic diseases. Sample should be subcultured from primary media (BA& MA) in alternative days especially when enteric fever is suspected. Expected isolates include S.aureus, Salmonella, E.coli, Brucella and few fungi like candida when immunosuppression is there.

Anaerobic blood cultures are not available routinely.

Rapid Culture methods like Bactec or Bactalert provide early indications about bacterial growth in the inoculum which may be confirmed by manual culture techniques

### **Sputum**

Sputum is the material from the lower respiratory infections most commonly submitted for bacteriological examination. The sputum is a mixture of bronchial secretions and inflammatory exudates coughed up into the mouth and expectorated. For Collection, Instruct patient to take a deep breath and cough up sputum directly into a wide-mouth screw capped plastic sterile container of 50 - 100 ml capacity (avoid saliva or postnasal discharge). Ideal to collect when patient wakes up and with first cough. On several occasions repeat sample may be required to isolate the causative agent.

A direct gram smear of sputum sample is having critical value. But if the number of epithelial cells is more, the sample is rejected. Inoculate the purulent part of the sample in to BA, MA and CA. Possible Pathogens are Strep. Pneumonia, Klebsiella, Haemolytic Steptococci and H. influenza.

### **CSF**

Lumbar puncture is used to collect the CSF for examination. CSF is usually collected by a Physician trained in procedure with aseptic precautions. Only 3-5 ml CSF is collected into a labeled sterile containers. The collected specimen of CSF should be despatched promptly to the laboratory, delay may cause death of delicate pathogens, eg. Meningococci. It is important when there is delay in transportation of specimens to Laboratory do not keep in Refrigerator, which tends to kill H. Influenza. If delay is anticipated leave at Room Temperature

Direct microscopy either by a wet mount or by Gram's staining will help to reveal the aetiology whether is that of bacterial or viral origin. Inoculate in to BA, CA & MA. If H. influenza is suspected streak S. aureus colonies across the plate to demonstrate satellitism. Possible pathogens include Strep. Pneumonia, Haemophilus influenza, E. coli, Klebsiella etc.

### **Stool**

Freshly passed stool samples is recommended, avoid specimens from a bed pan. Use clean container. Fresh stool unmixed with urine is collected in clean, dry and sterile container. Refrigerate at 4°C if necessary; do not freeze. A rectal swab is unsatisfactory, unless it is heavily charged and visibly stained with faeces collected from rectum, not anus. Whenever possible, a specimen of faeces should be

collected. The specimen is collected into 25 ml screw capped wide mouthed disposable container. Collect 1-2 ml of faeces, and apply the cap tightly.

Take care not to soil the rim or outside of the bottle. Transmit the container quickly to laboratory.

Specimen is inoculated into MA. As selective media DCA, XLD or BSA can be used. TCBS & alkaline peptone water is used in suspected cases of cholera if the stool sample has a rice water appearance. Sel. F Broth is the most commonly used Enrichment media. Possible pathogens include Pathogenic strains of E. Coli, salmonella, Shigella, Vibrio etc.

### **Pus swabs**

If material cannot be obtained with a needle and a syringe, a swab must be used. Swabs can be collected from wound, throat, nose, ear, eye, cervix, vagina, etc. The swab should then be inoculated onto appropriate culture media as soon as possible after collection or it can be placed immediately into a suitable transport medium (eg. Amies or Stuart's). Dry swabs are unacceptable.

A direct Gram smear of pus sample is done. Inoculate the pus sample in to BA, MA and CA, RCM ( if anaerobic organism suspected) and incubated at 37°C The organism grown is identified. Possible Pathogens are Strep. pyogenes, Staph aureus, Klebsiella, coliform bacilli etc.

### **Antibiotic sensitivity test**

The introduction of various antimicrobials for treating variety of infections showed the necessity of performing antimicrobial susceptibility testing as a routine procedure in all microbiology laboratories. In laboratories it can be made available by using antibiotic disk which will diffuse slowly into the medium where the suspected organism is grown. Various chemical agents such as antiseptics, disinfectants, and antibiotics are employed to combat with the microbial growth. Among these, antibiotics are generally defined as the substances produced by the microorganism such as Penicillium, which has the ability to kill or inhibit the growth of other microorganisms, mainly bacteria. Antimicrobial susceptibility tests (ASTs) basically measures the ability of an antibiotic or other antimicrobial agent to inhibit the invitro microbial growth.

There are many different procedures that microbiologists use to study the effects of various antimicrobial agents in treating an infection caused by different microorganisms. Mueller Hinton Agar is considered as best medium for the routine susceptibility testing. Fastidious organisms which require specific growth supplements need different media to grow for studying the susceptibility patterns.

The Kirby Bauer test is a qualitative assay whereby disks of filter paper are impregnated with a single concentration of different antibiotics or any chemicals that will diffuse from the disk into the agar. The selected antibiotic disks are placed on the surface of an agar plate which has already been inoculated with test bacteria. During the incubation period, the antibiotics/chemicals diffuse outward from the disks into the agar. This will create a concentration gradient in the agar which depends on the solubility of the chemical and its molecular size. The absence of growth of the organism around the antibiotic disks indicates that, the respected organism is susceptible to that antibiotic and the presence of growth around the antibiotic disk indicates the organism is resistant to that particular antibiotic. This area of no growth around the disk is known as a zone of inhibition, which is uniformly circular with a confluent lawn of growth in the media

The diameters of the zone of inhibition are measured (including disk) using a metric scale. The measured zone diameter can be compared with a standard chart for obtaining the susceptible and resistant values. There are zone of intermediate resistance which means that the antibiotic may not be sufficient enough to eradicate the organism from the body.



ABST plate

### Common Bacterial Diseases

#### Urinary tract infection

UTI is an infection that affects the urinary tract. Symptoms from a lower urinary tract include pain with urination, frequent urination, and feeling the need to urinate despite having an empty bladder. The most common cause of infection is *Escherichia coli*, though other bacteria or fungi may rarely be the cause. Risk factors include female anatomy, sexual intercourse, diabetes, obesity, and family history. a urine culture may be useful.

Diagnosis by urine culture ,microscopy for the presence of white blood cells, red blood cells and bacteria. A method of collecting urine called "clear catch" is used, which is where a person first washes their genital area before collecting a urine sample mid-flow. This helps to prevent bacteria from around the genital area getting caught in the sample.

#### Syphilis

systemic disease that is caused by the spirochaete *Treponema pallidum*. Syphilis is usually a sexually transmitted disease, but it is occasionally acquired by direct non

sexual contact with an infected person, and it can also be acquired by an unborn foetus through infection in the mother.

The most common laboratory procedures for the diagnosis of syphilis, are based on detection of syphilis reagin (an antibody-like substance) by initiating its reaction with an antigen to produce visible clumping, or flocculation, within the serum. Widely used among them are the RPR (rapid plasma reagin) test and the VDRL (venereal disease research laboratory) test; both are rapid techniques with a relatively high degree of sensitivity and specificity. However, none of the flocculation tests is fully specific for syphilis; "false positive" results are common in a wide variety of infections, from mononucleosis to malaria and even influenza. For this reason, positive RPR and VDRL tests are routinely followed by other more complicated and expensive Treponemal serological blood tests in order to detect *T. pallidum* directly. In addition, the spirochetes can be viewed by examining fluid from the primary chancre or secondary lesions under the microscope.

### **Typhoid fever**

It is an acute illness associated with fever caused by the *Salmonella typhi* bacteria. It can also be caused by *Salmonella paratyphi*, a related bacterium that usually causes a less severe illness. The bacteria are deposited in water or food by a human carrier and are then spread to other people in the area. The ingestion of contaminated food or water, the *Salmonella* bacteria invade the small intestine and enter the bloodstream temporarily. The bacteria are carried by white blood cells in the liver, spleen, and bone marrow, where they multiply and reenter the bloodstream. People develop symptoms, including fever,

Diagnosis of typhoid fever is made when the *Salmonella* bacteria are detected with a blood and stool culture. Typhoid fever is treated with antibiotics and symptoms are poor appetite, headaches, generalized aches and pains, fever, and lethargy. Approximately 3%-5% of patients become carriers of the bacteria after the acute illness.

### **Tuberculosis (TB)**

It is an infection caused by slow-growing bacteria that grow best in areas of the body that have lots of blood and oxygen. That's why it is most often found in the lungs. This is called pulmonary TB. Diagnosis of pulmonary TB is made by sputum smear examination and culture. But TB can also spread to other parts of the body, which is called extrapulmonary TB. Treatment is often a success, but it is a long process. It usually takes about 6 to 9 months to treat TB. But some TB infections need to be treated for up to 2 years.

## **Cholera**

It is an acute infection of the small intestine caused by the bacterium *Vibrio cholerae* and characterized by extreme diarrhea with rice water stool . It causes rapid and severe depletion of body fluids and salts which can be fatal and may lead to the death of the patient.

## **Bacterial Meningitis**

Bacterial meningitis is an extremely serious illness that requires immediate medical care. If not treated quickly, it can lead to death within hours -- or lead to permanent damage to the brain and other parts of the body.

The most common bacteria causing meningitis in children and adults are *Neisseria meningitidis* ("meningococcus"), *Streptococcus pneumoniae* ("pneumococcus") and, in older patients with decreased immunity, *Listeria monocytogenes*. *Haemophilus influenzae* type b (Hib) was a common cause of meningitis in infants and young children until the Hib vaccine was introduced for infants. Vaccines are available for both *Neisseria meningitidis* and *Streptococcus pneumoniae*. They are recommended for all children and adults at special risk.

## **Dysentery**

Dysentery is characterised by loose stools with presence of blood and mucus. It is caused by bacteria like *Salmonella*, *Shigella*, and also by the parasite *Entamoeba histolytica*.

## **Tetanus**

Caused by *Clostridium tetani* which is a toxin mediated infection. Usually transmitted by spores in the dust or soil.

## **Leptospirosis**

The disease Leptospirosis is caused by the spirochaete *Leptospira* and is characterised by fever and associated jaundice.

## **Serological diagnosis of bacterial diseases**

### **WIDAL TEST**

Widal test is a tube agglutination test employed in the serological diagnosis of enteric fever.

Principle: Patients' suffering from enteric fever would possess antibodies in their sera which can react and agglutinate serial doubling dilutions of killed, coloured *Salmonella* antigens in a tube agglutination test.

Requirements: mainly 4 types of antigens are used

S.typhi O antigen,

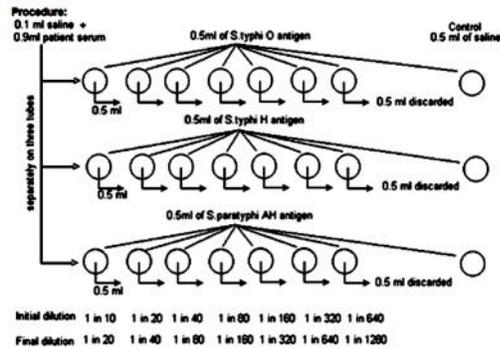
S.typhi H antigen,

S.paratyphi AH antigen and optionally

S.paratyphi BH antigen.

Patient serum is doubly diluted by mixing and transferring from 1:10 to

1:640 in three-four rows. First row usually comprises of Felix tubes, where somatic S.typhi O antigen is added. For all the remaining rows, Dreyer's tubes are taken; where different flagellar H antigens are added. Each tube must contain 0.5ml of diluted serum. A test tube with only saline is kept in each row as control. All the tubes (including control) in a row are mixed with 0.5ml of antigen suspension. The first row is treated with S.typhi O antigen, the second row with S.typhi H antigen, the third row with S.paratyphi AH antigen and the fourth row with S.paratyphi BH antigen.



After all the tubes have been treated with specific antigen suspensions, the Widal rack is placed in a thermo statically controlled water bath maintained at 37oC for overnight incubation. Another approach is to incubate the tubes at 50-55C.

### Reading the results:

The control tubes must be examined first, where they should give no agglutination. The agglutination of O antigen appears as a "matt" or "carpet" at the bottom. Agglutination of H antigens appears loose, wooly or cottony. The highest dilution of serum that produces a positive agglutination is taken as titre. The titres for all the antigens are noted.

**Slide widal test:** A slide widal test is more popular among diagnostic laboratories as it gives rapid results.

**Qualitative test:** One drop each of undiluted patients' serum samples for the four antigens are placed on the circled card and one drop of each of the four Salmonella antigens are added separately and gently rotated for one minute. Appearance of agglutination gives qualitative results.

**Quantitative test:** To know the titre for each of the antigens, the test is repeated with dilutions of serum.

**Interpretation of widal test:**

Timing of test is important, as antibodies begin to arise during the end of first week. The titres increase during second, third and fourth week after which it gradually declines. The test may be negative in early part of first week. Single test is usually of not much value. A rise in titre between two sera specimens is more meaningful than a single test. If the first sample is taken late in the disease, a rise in titre may not be demonstrable. Instead, there may be a fall in titre. Baseline titre of the population must be known before attaching significance to the titres. The antibody levels of individuals in a population of a given area give the baseline titre. A titre of 100 or more for O antigen is considered significant and a titre in excess of 200 for H antigens is considered significant.

Patients already treated with antibiotics may not show any rise in titre, instead there may be fall in titre.

Patients who have received vaccines against Salmonella may give false positive reactions. This can be differentiated from true infection by repeating the test after a week. True untreated infection results in rise in titre whereas vaccinated individuals don't demonstrate any rise in titre. Those individuals, who had suffered from enteric fever in the past, sometimes develop anti-Salmonella antibodies during an unrelated or closely related infection. This is termed anamnestic response and can be differentiated from true infection by lack of any rise in titre on repetition after a week.

**Rapid Plasma Reagin (Card Test)**

RPR card test is a macroscopic, nontreponemal flocculation card test used to screen syphilis. The antigen is prepared from a modified Venereal Disease Research Laboratory (VDRL) antigen suspension containing choline chloride to eliminate the need to heat inactivate serum, ethylenediaminetetraacetic acid (EDTA) to enhance the stability of the suspension, and finely divided charcoal particles as a visualizing agent. In the test, the RPR antigen is mixed with unheated or heated serum or with unheated plasma on a plastic coated card.

The RPR test measures IgM and IgG antibodies to lipoidal material released from damaged host cells as well as to lipoprotein like material, and possibly cardiolipin released from the treponemes.

The antilipoidal antibodies are diseases, but also produced in response to nontreponemal diseases of an acute and chronic nature in which tissue damage occurs.

If antibodies are present, they combine with the lipid particles of the antigen, causing them to agglutinate. The charcoal particles coagglutinate with the antibodies and show up as black clumps against the white card. If antibodies are not present, the test mixture is uniformly gray. Without some other evidence for the diagnosis of syphilis, a reactive nontreponemal test does not confirm *T. pallidum* infection.

Serum and plasma are both suitable specimens for the qualitative test; however serum is the preferred sample for the quantitative test. Test plasma samples within 48 hrs after collection.

Rapid Plasma Reagin Test is a general screening test, can be adapted to automation. But it cannot be performed on CSF. Cardiolipin antigen is modified with choline chloride to make it more stable attached to charcoal particles to allow macroscopic reading, antigen comes prepared and is very stable

Test Procedure: Serum or plasma added to circle on card and spread. One drop of antigen is added. Rotate at 100 rpm/minute for 8 minutes. Results are read macroscopically. A control must be run. RPR appears to be more sensitive than the VDRL.

### **Anti-streptolysin O (ASO Test)**

Anti-streptolysin O is the antibody made against streptolysin O. Streptolysin O: is an immunogenic, hemolytic toxin produced by most strains of group A and many strains of groups C and G streptococci. The O in the name stands for oxygen-labile.

#### **Procedure:**

The reagent and sample are both allowed to reach room temperature.

On separate slides, a drop of sample, positive control and negative control is added.

Then a drop of the latex reagent should be added to each. Rotate the card for proper mixing for 1-2 minutes and then observe agglutination i.e. small clumps formation. Agglutination indicates a positive ASO test. To find the titre, the test is repeated with dilutions of serum.

### **Card tests (Immuno chromatography)**

Immuno chromatographic assays, also called lateral flow tests or simply strip tests or card test

These features make strip tests ideal for applications, rapid point of care testing. Some of the more common lateral flow tests currently on the market are tests for *Leptospira*, *Streptococci*, *Chlamydia* etc:

## Unit 4.1.8 Laboratory Diagnosis of Common viral diseases

### Introduction

Viruses are obligate intracellular parasites. They do not have a cellular organization and contain only one type of nucleic acid, either DNA or RNA (DNA viruses or RNA viruses) but never both. They are small size (20-300 nm in diameter) The protein shell, or coat, that encloses the nucleic acid genome and mediates the attachment of the virus to specific receptors on the host cell surface is called capsid. Naked viruses are composed of nucleic acid + capsid (nucleocapsid). The outermost layer, called envelope is a lipoprotein membrane composed of lipid derived from the host cell membrane and protein that is virus-specific (Enveloped viruses and non-enveloped viruses). The whole virus particle is called virion.

The medical importance of viruses lies in their ability to cause a very large number of human diseases. Viral diseases range from minor ailments like common cold to terrifying diseases like rabies and AIDS. A number of viruses are suspected of causing cancer in animals, including humans, and are frequently referred to as oncogenic viruses

### Common Viral diseases

**AIDS** The fatal illness caused is by HIV (human immunodeficiency virus). This infection is transmitted through blood and blood products and through sexual contact. It can lead to severe immunosuppression. The disease is diagnosed by ELISA and confirmed by western Blot test

**Viral Hepatitis:** This inflammation of the liver is caused by 5 viruses namely, hepatitis A, B, C, D and E. Each of these has its own symptoms ranging from jaundice, low immunity and cirrhosis. It can be contracted through sexual contact, through blood (Hepatitis B, C, D) food and water (Hepatitis A & E). Diagnosis is by detection of antigen (HBsAg) or antibody through serology.

**Rabies:** This is one of the fatal diseases caused by rabies virus, which is evolved from infected animal bites (zoonosis). Characteristic symptom is hydrophobia. Diagnosis technique used is immunofluorescence. Vaccination is effective.

**Influenza:** The influenza virus affects large populations annually cause epidemics and pandemics. Transmitting through aerosols.

**Measles:** Commonest childhood infection. It is a children's infection that can be prevented by a vaccine (MMR).

**Mumps** The mumps is a contagious viral infection that affects the salivary glands. MMR vaccine is effective

**Dengue Fever** it is a mosquito born disease caused by dengue virus and is transmitted by, the *Aedes aegypti* mosquito. It may lead to a more serious form of the disease, dengue hemorrhagic fever (DHF), Chikungunya is another viral disease transmitted by same type of mosquito

### **Diagnostic tests for viral infections**

In general, diagnostic tests can be grouped into 3 categories:

direct detection,

indirect examination (virus isolation),

serology.

In direct examination, the clinical specimen is examined directly for the presence of virus particles, virus antigen or viral nucleic acids. In indirect examination, the specimen is introduced into cell culture, eggs or animals in an attempt to grow the virus: This is called virus isolation,

A serological diagnosis can be made by the detection of rising titres of antibody between acute and convalescent stages of infection, or the detection of IgM. In general, the majority of common viral infections can be diagnosed by serology

### **Direct Examination of Specimen**

1. Electron Microscopy morphology / immune electron microscopy
2. Light microscopy histological appearance - e.g. inclusion bodies
3. Antigen detection immunofluorescence, ELISA etc.
4. Molecular techniques for the direct detection of viral genomes (PCR)

### **Indirect Examination**

1. Cell Culture - cytopathic effect, haemadsorption, confirmation by neutralization, interference, immunofluorescence etc.
2. Pocks on CAM - haemagglutination, inclusion bodies
3. Animals disease or death confirmation by neutralization

### **Serology**

The diagnosis of viral infections by detection of specific antiviral antibodies is a traditional method whose clinical utility is limited by the need for comparison of acute and convalescent antibody titers. However, detection of virus-specific IgM

antibodies allows a diagnosis to be made from a single specimen. Viruses for which detection of virus-specific IgM antibodies are useful include EBV (IgM antibodies to the viral capsid antigen); CMV; hepatitis A virus; hepatitis B virus (IgM antibodies to the hepatitis B core antigen); parvovirus B19; measles, rubella, and mumps viruses; Commonest serological methods including different types of ELISA, Immunochromatography Hemagglutination, blot tests etc.

### 4.1.9 Laboratory Diagnosis of Common Parasitic diseases

#### Introduction

**Parasitism** is a form of living in which two different organisms co-exist over a long time. There are several different outcomes of parasitism. When a parasite lives with another organism, this can cause **commensalism** which is defined as two organisms that co-exist and one of the organisms benefit from the other while neither harming nor helping the other organism. Parasitism may be '**Mutualism**' in which both organisms benefit without causing any injury to one another. Another form of parasitism would be the parasite, which is usually a smaller organism benefits from the other organism called the host and here the host is usually harmed.

Medical Parasitology is the branch of medical science dealing with parasites, which live within the human body. The parasites affect several billions of people worldwide, kill millions annually and cause debilitating injuries such as blindness and disfigurement. World Health Organization estimates that one person in every four harbours parasitic worms.

Hosts are classified according to their role in the life cycle of the parasite into two types

- Definitive host that harbours the adult stage of the parasite or in whom sexual reproduction occurs
- Intermediate host that harbours larval stage of the parasite or in whom asexual reproduction occurs.

Vector is an organism which acts as a carrier and transmits the parasite from one another.

Zoonotic diseases (Zoonosis) is a type of human disease naturally acquired from an infected organism.

There are different types of parasites. Human parasites are either unicellular (protozoa) or multicellular. The parasites may live inside the host (endoparasites) or on the host surface (ectoparasites).

Endoparasites are sub-classified into Helminthic parasites (multicellular organisms) and Protozoan parasites (unicellular organisms). Helminthic parasites are either flat worms (Trematodes), segmented ribbon like worms (Cestodes) or cylindrical worms (Nematodes).

According to the area of inhabitation in humans, the parasites are classified into intestinal and blood-borne parasites.

### **Blood Parasites**

Blood Parasites are the type of parasites which inhabits in the blood and tissues other than intestine. Common blood parasites include Plasmodium, Wuchereria bancrofti, Brugia Malayi, Trypanosoma, leishmania etc.

Microscopy remains the cornerstone of the laboratory diagnosis blood parasite infections. Examination of thick and thin peripheral blood smears stained with any of the Romanowsky group of stains is the accepted method for the detection and identification of species of Plasmodium, Brugia and Wuchereria.

### **Malaria & Its Lab Diagnosis**

Malaria is the most important tropical disease known to man. It remains a significant problem in many tropical areas. Malaria is spreading as a result of environmental changes, including global warming, civil disturbances, increasing travel and drug resistance. There are approximately 100 million cases of malaria worldwide. Malaria is caused by protozoa of the Plasmodium species. There are four species which infect both humans and animals; Plasmodium malariae (quartan malaria), Plasmodium vivax (benign tertian malaria), Plasmodium falciparum (malignant tertian malaria, subtertian malaria) and Plasmodium ovale (ovale tertian malaria). Malaria is transmitted by the female anopheline mosquito.

Lifecycle of malaria comprises of an exogenous sexual phase (sporogony) with multiplication in Anopheles mosquitoes and an endogenous asexual phase (schizogony) with multiplication in the vertebrate host (Man). The latter phase includes the development cycle in the red cells (erythrocytic schizogony) and the phase taking place in the parenchymal cells of liver (pre-erythrocytic schizogony).

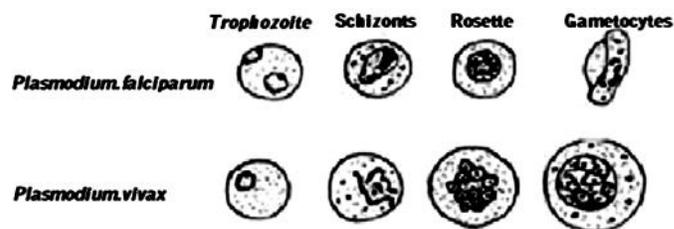
When a female Anopheles mosquito bites an infected person, it ingests blood which may contain the mature sexual cells (male and female gametocytes) which undergo

a series of developmental stages in the stomach of the mosquito. Exflagellation (the extrusion of rapidly waving flagellum-like microgametes from microgametocytes) occurs resulting in the production in a number of male and female gametes. Fertilization occurs producing a zygote which matures to an ookinete. This penetrates the stomach wall of the mosquito where it grows into an oocyst and it further matures to become a motile sporozoite.

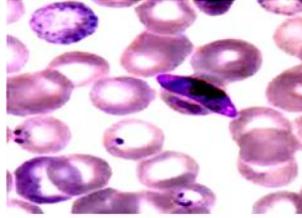
The sporozoites migrate from the body cavity of the mosquito to the salivary glands and the mosquito now becomes infective. Sporozoites enter into the blood stream of a host when the mosquito feeds on blood. Following the inoculation, the sporozoites leave the blood and enter the parenchymal cells of the liver. In all four species, asexual development occurs in the liver cells, a process known as pre-erythrocytic schizogony, to produce thousands of tiny merozoites which are released into the circulation after about 2 weeks. Once in the circulation, the merozoites invade the red cells and develop into trophozoites. After a period of growth the trophozoite undergoes an asexual division, erythrocytic schizogony. When the mature trophozoite starts to divide in the red blood cell, separate merozoites are formed resulting in a schizont. When fully developed, the schizont ruptures the red blood cell containing it, liberating the merozoites into the circulation. These merozoites will then infect new red cells and the process of asexual reproduction in the blood tends to proceed. Some of the merozoites entering red blood cells do not form trophozoites then schizonts but develop into gametocytes and this process takes place in deep tissue capillaries. This erythrocytic cycle of schizogony is repeated over and over again in the course of infection, leading to a progressive increase of parasitemia.

Clinical features of malaria are due to the liberation of fever-producing substances, especially during schizogony. The common features includes Fever of regular pattern, Haemolytic anemia, Splenomegaly and Jaundice. Jaundice may be severe in *P. falciparum* infection

The Lab diagnosis of malaria infection is still based on finding different stages of plasmodium spp in blood films. In thin films the red blood cells are not distorted so the morphology of the parasitized cells can be seen. Species identification can be made, based upon the size and shape of the various stages of the parasite and the presence of stippling (i.e. bright red dots) and fimbriation (i.e.



ragged ends). However, malaria parasites may be missed on a thin blood film when there is a low parasitemia. Therefore, examination of a thick



blood film is recommended. With a thick blood film, the red cells are approximately 6-20 layers thick which results in a larger volume of blood being examined.

### Other methods of Malaria Diagnosis

#### QBC technique

The QBC technique a rapid and sensitive test designed to simplify malaria diagnosis by enhancing microscopic detection of parasites. This method involves staining parasite deoxyribonucleic acid (DNA) in micro-hematocrit tubes with fluorescent dyes, e.g. acridine orange, and its subsequent detection by fluorescent microscopy. Finger-prick blood is collected in a hematocrit tube containing acridine orange and anticoagulant. The tube is centrifuged at 12,000 g for 5 min and immediately examined using an fluorescent microscope. Parasite nuclei fluoresces bright green, while cytoplasm appears yellow-orange

Recently QBC technique is the preferred diagnostic method over light microscopy probably because of increased sensitivity at low parasitemia. Although the QBC technique is simple, reliable, and user-friendly, it requires specialized instrumentation, is more costly than conventional light microscopy, and is poor at determining species and numbers of parasites.

Various Serological methods are now available in the malaria diagnosis and increasingly used in the laboratories. Which includes immunochromatographic cards termed Rapid malaria tests (RDTs) , immunofluorescent antibody assays and molecular techniques like PCR and Flowcytometry etc.

### Filariasis & Its Lab Diagnosis

*Wuchereria bancrofti* and *Brugia malayi* are the important nematodes causing lymphatic filariasis. the adult worms residing inside the lymphatics, , obstructs the flow of lymph causing swelling and fibrosis of the affected area (elephantiasis). Filariasis is transmitted by the mosquito. they are ovo-viviparous ie giving birth to living embryos. The larva of filarial worm is called as microfilaria. The microfilaria of *W.bancrofti* is colourless and transparent with blunt head and pointed tail. It is covered by a hyaline sheath which projects slightly beyond the extremities. The central axis

of the body has granules or nuclei which do not extend upto the tail. It serves as a distinguishing character to identify the species. The microfilaria of *Brugia malayi* is smaller and tail tip is not, free from nuclei.

The life cycle of filarial worms completed in two hosts in man and Mosquitos belong to the genus of *Culex anopheles* or *Aedes*. Microfilariae enter the host during a blood meal when the vector, a mosquito, punctures the skin. The infective larvae enter through the wound and migrate to the peripheral lymphatics where they grow to mature male and female worms. They can live there for several years. After mating, the gravid females release sheathed microfilariae into the peripheral blood where they can be detected 8-12 months after the initial infected bite. The mosquito acquires the infection by ingestion of the microfilaria in the blood meal. The microfilariae lose their sheath on arrival in the stomach of the mosquito due to gastric juices. The larvae migrate to the thoracic muscles and develop into infective larvae over a period of 6-14 days. The larvae then migrate to the mouthparts of the mosquito which infects the host during a blood meal.

The blood stages of filariae, microfilariae, vary in times when they are present in the peripheral blood, corresponding with the peak biting time of the vector. Thus, in nocturnally periodic forms the microfilaria are present in the peripheral blood circulation at night; during the day they reside in the deep tissues, particularly the lungs.

Lab diagnosis of filariasis involves examination a thin and thick blood smears blood sample for filarial detection is ideally collected between 10 pm to 2 am because of the nocturnal habit of microfilaria.

There are 4 characteristics that are generally used in diagnosing microfilaria:

- The presence or absence of a sheath.
- The presence or absence of nuclei in the tip of the tail.
- The innerbody - can or cannot be demonstrated.
- The size of the microfilaria.

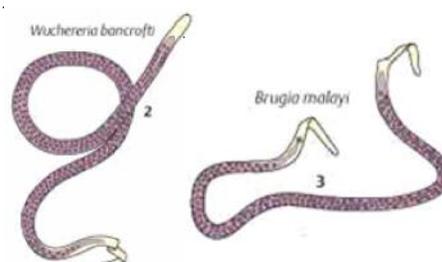


Figure showing Microfilaria of *W.bancrofti* & *B.malayi*

### Methods:

#### Wet preparation

Two or three drops of blood are taken in a clear glass slide and put a cover slip on it. Examine under low power objective. The microfilaria can be seen wriggling about in the blood in unstained preparation.

### Stained preparation

A thick blood smear is prepared and dehaemoglobinised by putting the slide in distilled water for 10 minutes. Air dry, stain with leishman's stain or Methylene blue. Alternatively JSB or Field's stains are used.

### Concentration Method

The concentration can be done by taking a large quantity of blood (5-10ml) and adding 10ml. of 2% acetic acid or distilled water, to lyse the RBCs. The blood is then centrifuged and smear is prepared from the sediment. The smears are air dried, dehaemoglobinised, stained and examined for the presence of parasite. Filariasis can also be diagnosed indirectly by serological tests.

### Intestinal Parasites

Parasites inhabiting the intestinal tract are grouped as intestinal parasites. Among parasitic infections, intestinal parasites are the most prevalent infections in humans in developing countries. They cause a significant morbidity and mortality in endemic areas. Spread of these parasites mostly occurs through faecal contamination as a result of poor sewage and poor quality of water. Food and water-borne outbreaks often reported.



Intestinal parasites may be classified into Helminths (multicellular) and Protozoans (single celled) most common helminths are *Ascaris lumbricoides* (roundworm), *Trichiuris trichiuria* (whipworm), *Ancylostoma duodenale*, and *Necator americanus* (hookworms).

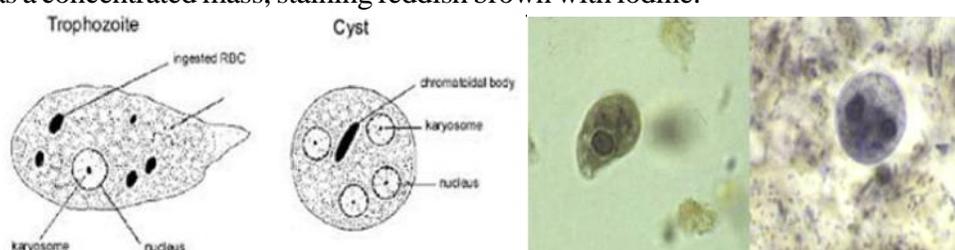
*Entamoeba histolytica* is the most common intestinal protozoan parasite, Amoebiasis is the third leading cause of death from parasitic diseases worldwide, with its greatest impact on the people of developing countries.

Laboratory diagnosis of intestinal parasite infections involves examination of faecal sample. Trophozoite and cyst are the stages of protozoan parasites usually found in stool. Trophozoite stages are most often found in watery or diarrhoeic faecal specimens and usually cysts are not seen in such specimens. On the other hand, cysts are the stage typically found in formed faecal specimens. A mixture of trophozoites and cysts may occur in softer and semi-formed faeces. In direct smears of faeces in saline, motile trophozoites may be found. Microscopic examination

reveals motile trophozoites containing red blood cells of *E. histolytica*. cysts can be identified by the presence of brown coloured glycogen mass in yellow coloured cytoplasm with chromatoid bars. Eggs and larvae are the stages of helminths usually found in stools, though whole adult worms or segments of worms may also be seen.

### Morphology of Trophozoite & Cyst of *E. histolytica*

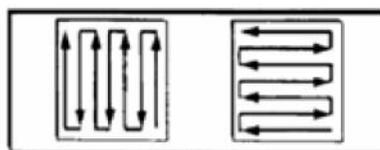
The trophozoite of *E. histolytica* in fresh dysenteric stools exhibit ingested red blood cells and clear pseudopodia. They can be up to 60 $\mu$ m in size and motility is rapid and unidirectional. Cyst of *E. histolytica* is 10-15 $\mu$ m in size and contain one to four nuclei. Chromatoid bodies are usually present in young cysts as elongated bars with bluntly rounded ends. Glycogen is usually diffuse, but in young cysts it is often present as a concentrated mass, staining reddish brown with iodine.



Saline mount, iodine mount or concentration techniques are employed in the examination of stool for detection of various intestinal parasites. Flootation and sedimentation faecal concentration procedures are more useful for detecting ova or eggs of helminths.

### Saline & Iodine Mount

1. Take a clean glass slide and label
2. Put a drop of saline in the center of the left half of the slide and a drop of iodine solution in the right half of the slide
3. With an applicator stick, pick up a small portion of faecal specimen and mix the drop of saline. Similarly take a small amount of the specimen and mix with the drop of iodine, to prepare an iodine mount.
4. Cover the drop of saline and iodine with a cover glass
5. Place the glass slide in the stage of microscope and focus with low power objective, examine the entire cover glass area in a systematic manner. When an organism or suspicious material is seen, change to high power to observe the detailed morphology.



**Concentration methods:**

Direct microscopy is useful for the observation of motile protozoan trophozoites and due to the low density of the parasites in the faeces, is not recommended solely for the routine examination of suspected parasitic infections. To increase the probability of finding the parasites in faecal samples, concentration methods are preferred. Stool concentration techniques are of two types. Formalin- Ether (Formalin- Ethyl acetate) Sedimentation method and Flotation Method (Using Zn SO<sub>4</sub> or Saturated NaCl Solution)

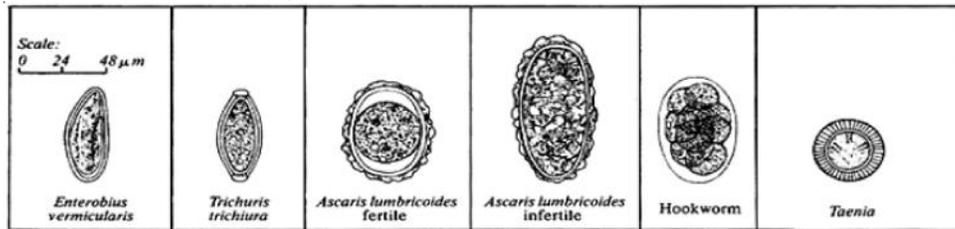


Figure showing the Common Eggs found in stool

**DETAILING OF PRACTICALS**

**Demonstration of Operation procedure of hot air oven**

Hot air oven is operated and procedure is demonstrated and is recorded in the practical log

**Demonstration of operation procedure of Autoclave**

Hot air oven is operated and procedure is demonstrated and is recorded in the practical log

**Preparation of Peptone water**

Composition

- Peptone 10 gm
- Sodium chloride 5 gm
- Distilled water 1000ml

Dissolve the ingredients in D water

Adjust the pH to 7.5, Dispence into tubes and sterilize by autoclaving at 121<sup>o</sup>c 15 minutes

**Uses:**

1. Basis for fermentation tests.
2. To test Indole production
3. To study motility

**Preparation of Nutrient Agar:**

Nutrient Agar is a general purpose, nutrient medium used for the cultivation of microbes supporting growth of a wide range of non-fastidious organisms. Nutrient agar is popular because it can grow a variety of types of bacteria and fungi, and contains many nutrients needed for the bacterial growth

**Composition**

Peptone	10 gm
Sodium chloride	5 gm
Meat extract	10 gm
Agar	20 gm
Distilled water	1000ml

Mix the ingredients and dissolve by gentle heat in Distilled water, Adjust the pH 7.5. sterilize by autoclaving at 121<sup>o</sup>c 15 minutes. Allow to cool to 50<sup>o</sup>c. Pour into sterile Petri dishes or tubes.

**Preparation of Blood Agar****Composition.**

1. Sterile defibrinated blood 5-10 %
2. Melted agar
3. Beef extract
4. Peptone water
5. NaCl

Prepared by adding 5-10% sterile Blood into Nutrient Agar at 50<sup>o</sup>c. Sheep blood is commonly used.

Dispense into tubes and sterilize by autoclaving at 121<sup>o</sup>c 15 minutes. Pour into Petri dishes

**Preparation of Chocolate Agar**

Nutrient Agar is melted and cooled to 75<sup>o</sup>c. Add 5-10 % blood. Mix well and the

medium become chocolate brown in colour. The media is cooled to at 50°C. Pour into Petri dishes.

Preparation of MacConkey Agar

### Composition

1. Peptone	20 gm
2. Sodium taurocholate	5 gm
3. Agar	20 gm
4. Lactose	10 mg
5. Neutral Red Solution 2%	3.5 ml
6. Distilled Water	1000 ml

Dissolve peptone and sodium taurocholate in water by heating. Add agar and autoclave. Adjust pH to 7.5. Add Lactose and Neutral red and autoclave. Dispense into plates.

### Handling, sterilisation and use of bacterial wire loop

#### Demonstration of inoculation methods

Various inoculation methods are demonstrated and is recorded in practical log

#### Inoculation of Liquid Culture media

#### Demonstration of hanging drop method of motility

1. With a toothpick, spread Vaseline or Petroleum jelly on the four corner of a clean coverslip.
2. After thoroughly mixing one of the cultures, use the inoculating loop to aseptically place a small drop of the bacterial suspension in the center of a coverslip
3. Lower the cavity slide, with the concavity facing down, onto the coverslip so that the drop protrudes into the center of the concavity of the slide. Press gently to form a seal.
4. Turn the hanging drop slide over and place on the stage of the microscope so that the drop is over the light hole. Examine the drop by first locating its edge under low power and focusing on the drop. Switch to the high-dry objective (40 x). To increase the contrast and to see the bacteria clearly, close the diaphragm as much as possible.
5. Distinguish between actual motility and Brownian movement
6. Discard the coverslips and any contaminated slides in a container with disinfectant solution.

**Demonstration of colony characters of commonest pathogens****Preparation of bacterial smear- from liquid media****Preparation of bacterial smear- from solid media****Perform simple staining****Perform Gram staining****Preparation of the smear**

Grease or oil free slides are essential for the preparation of microbial smears. Label the slide. Drawing a circle on the underside of the slide using a glassware-marking pen may be helpful to clearly designate the area in where the smear is prepared. With a sterile cooled loop, place a loopful of the broth culture on the slide or pick up a very small sample of a bacterial colony and gently stir into the drop of water/saline on the slide to create an emulsion and spread (It is very important to prevent preparing thick, dense smears which contain an excess of the bacterial sample.) Heat fixing kills the bacteria in the smear, firmly adheres the smear to the slide, and allows the sample to more readily take up stains. Allow the smear to air dry. Hold the slide at one end and pass the entire slide through the flame of a Bunsen burner two to three times with the smear-side up.

**Gram Staining Procedure**

1. Place slide with heat fixed smear on staining tray.
2. Gently flood smear with crystal violet and let stand for 1 minute.
3. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
4. Gently flood the smear with Gram's iodine and let stand for 1 minute.
5. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. The smear will appear as a purple circle on the slide.
6. Decolorize using 95% ethyl alcohol or acetone. Tilt the slide slightly and apply the alcohol drop by drop for 5 to 10 seconds until the alcohol runs almost clear. Be careful not to over-decolorize. Immediately rinse with water.
7. Gently flood with safranin to counter-stain and let stand for 45 seconds.
8. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
9. Blow to dry the slide with tissue paper.

View the smear using a light-microscope under oil-immersion.

## Results

Gram positive bacteria - violet /Purple

Gram negative bacteria - pink

### **Perform AFB staining**

(Ziehl- Neelsen method)

1. Prepare a smear of sputum on a clean glass slide, air dry and heat fix.
2. Flood the entire surface of slide with Ziehl-Neelsen 1% Carbol-fuchsin solution.
3. Heat slide slowly until steam arises. Maintain steaming for 5 minutes by using intermittent heat. (In no case must the stain solution boil or dry during staining process.)
4. Rinse slide in a gentle flow of water until all free stain is washed away. Drain off excess rinse water by tilting the slide.
5. Flood the slide with the decolorizing solution (20% Sulphuric acid) for 3 minutes. (If the slide is under decolorized after 3 minutes, further decolorize for 1 minute.)
6. Rinse the slide thoroughly with water. Drain off excess water from the slide. Wipe off back of slide with cotton soaked in decolorizer to clean the dried stains.
7. Rinse the slide again with water and drain off excess water from the slide by tilting the slide.
8. Flood the slide with 0.1% Methylene blue and counter-stain for 60 seconds.
9. Rinse the slide thoroughly with water and drain off any excess stain from the slide by placing the slide under gentle stream of running water and allow the smear to air dry.
10. Thoroughly examine the slide under oil immersion field of microscope, in a systematic manner for the presence of red coloured curved rod shaped organism in a blue back ground. Number of organisms noted in hundred oil immersion fields and graded as 1+ to 4+

### **Demonstration of ABST**

#### **Demonstrate and Perform RPR Test**

#### **Preparation of Thick & Thin Film smears for the diagnosis of blood parasites**

Blood smears should be prepared as soon as possible after collection (delay can result in changes in parasite morphology and staining characteristics).

**Thick Smear:**

1. Place a small drop of blood in the centre of the pre-cleaned, labelled slide.
2. Using the corner of another slide or an applicator stick, spread the drop in a circular pattern to get a thickness that a word to be barely read when placed it over a newsprint .
3. Lay the slides flat and allow the smears to dry thoroughly
4. Dehaemoglobinise the smear by placing the slide immersed in distilled water or diluted g.acetic acid solution and air dry
5. Do not fix thick smears with methanol or heat, Stain with Leishman's Stain or Field's stain.JSB stain is also preferred for staining blood smears.

**Thin smear:**

1. Place a small drop of blood on a clean, dry grease free glass slide,
2. Bring the spreader slide at a 30-45° angle up to the drop, allowing the drop to spread along the contact line of the 2 slides.
3. Quickly push the spreader slide toward the other end of the slide.
4. Make sure that the smears have a good feathered edge. This is achieved by using the correct amount of blood and spreading technique.
5. Allow the smears to dry in air. Fix the smears by dipping them in absolute methanol.
6. Stain with Leishman's stain or Field's stain.JSB stain is also preferred for staining blood smears.

**Demonstration of wet smear for filarial parasite**

**Perform direct smear of stool sample for parasites-saline and iodine**

**Demonstration of floatation/sedimentation Techniques for intestinal parasites**

### ASSESSMENT ACTIVITIES

- Chart showing Endemic infections of our part of world can be prepared
- An infection cycle with various routes may be plotted
- An album showing pioneers in microbiology may be prepared
- A comparison chart showing the characters of different microorganisms can be prepared
- A role play of students may be planned with scientists as characters telling their observations from findings and difficulties faced
- Bacterial models can be prepared by using plaster of Paris or themocol to show the cell structure.
- Comparison chart may be prepared for prokaryotic and eukaryotic comparison
- Preparation of a model showing the arrangements of bacteria using Beads and plastic balls .
- Student groups may be assigned to collect the name of antibiotics that are commonly used
- Chart showing Names of common blood & Intestinal parasites
- A comparison chart showing the different morphological forms of malarial parasites in blood film.
- A chart may be prepared on identifying features of common parasitic eggs found in stool sample.
- An album preparation on images of ova/eggs of common parasites found in stool.

### Theory Evaluation Questions

1. Among the following has an example for anaerobic bacteria. Find him  
(Staphylococcus, E.coli, M.tuberculosis, Clostridium) 1
2. Match the following
 

a) Basal media	Mac Conkey Agar
b) Enriched media	VR Fluid
c) Transport media	Peptone Agar
d) Indicator media	Blood Agar

(4x1=4)

- 3) A patient is suffering from UTI.  
Explain the steps of processing the sample ?  
Name two pathogens expecting from the sample? (4+2=6)
- 4) Antibodies are important in diagnosis.  
a) Name 4 types of antibodies  
b) What are the importance of antibodies in serological diagnosis?  
c) Draw the diagram of an antibody (2+2+2=6)
- 5) Spore formation and presence of capsule provide several advantages to a pathogenic bacteria.  
Name example for a bacteria that possess these structures (1+1=2)
- 6) An insect that transmit a disease is called ----- (1)
- 7) Different type culture methods are available for bacterial culture. Name four types bacterial culture? explain any one of them  
Add a note on anaerobic culture Add (2+2+=4)
- 8) Gram stain differentiates bacteria into two. What is basic principle of gram staining? explain the procedure of the same (2+3=5)
- 9) Prepare a short note about the following techniques  
b) ELISA      b)RPR      c) PCR (3x2=6)
- 10) A non Keralite employee suspected to have malaria approaches your OJT lab.  
What are the laboratory procedures used to diagnose his disease (3)

## Unit 4.2 Histotechnology & Cytology

Histological and Cytological techniques play a crucial role in the diagnosis of diseases based on the study of various morphological abnormalities of tissue and cells. Histopathological studies proved to be one of the most effective means in diagnosis of benign and malignant conditions of tissues. The unit describes various histological techniques adopted for the preparation of tissue Sections or smears for examination under microscope.

In histopathology, the tissue is taken off from the body by surgery, autopsy or biopsy then it is fixed over the glass slide and stained with dyes followed by examination under the microscope.

Cytological techniques helps to study the structure, function and the Chemistry of cells mainly for the microscopic interpretation of cells to detect cancer and other abnormalities. This includes the examination of samples collected from different parts of body

### Learning outcomes

#### The learner:

- identifies different types of specimens and the method of examination in histotechnology
- explains various steps in tissue processing
- identifies various specimens and processing techniques employed in cytology

### 4.2.1 Histotechnology -Introduction

Histology is the branch of science which deals with the gross & microscopic study of normal tissue of the body. Histopathology is the science which deals with the structural changes brought about in the human body by disease and identifying the disease and its cause. Histological technique deals with the processing and preparation of tissue for microscopic examination. The aim of good histological technique is to preserve microscopic anatomy of tissue, make them hard so that very thin section (4 to 5 micron) can be made for staining also should be possible. After staining, the section should represent the anatomy of the tissue as close to as possible to their structure in life So the pathologist can study them under the microscope.

Tissue for study can be obtained from:

- **Biopsies** - A medical diagnostic procedure in which cells or tissues are

removed from a patient and examined visually, usually with a microscope. The material for the biopsy may be obtained by aspiration through a needle, swabbing, scraping, or excision. The biopsy is a standard step in the diagnosis of both malignant and benign tumours and can also provide a wide range of other types of diagnostic information, particularly in connection with certain organs, such as the liver or the pancreas.

- **Autopsies**-Dissection and examination of a dead body and its organs and structures are called Autopsy. It is also called necropsy or postmortem examination. An autopsy may be performed to determine the cause of death, to observe the effects of disease, and to establish the evolution and mechanisms of disease processes.

### Methods of Examination of Tissue

- **Gross Examination**
- **Microscopic Examination**

Gross Examination is done to describe the specimen with respect to its consistency, appearance, discolouration and any apparent abnormalities. Microscopic examination can be examination of fresh specimens or examination of fixed specimens.

### Protocols followed in Histopathology

1. **Receipt & Identification**- Tissue specimen received in the laboratory have a request form that lists the patient information and history along with a description of the site of origin.
2. **Labeling of the specimen with numbering & Grossing** -The specimen are accessioned by giving them a number that will identify each specimen for each patient. The specimen is then cut into representative sections and is put in small plastic cassette to hold the tissue (Done by a pathologist or pathology assistant)
3. **Fixation** - It is a process in which a specimen is treated by exposing it to a fixative for a particular period of time in order to facilitate the succeeding steps. The purpose of fixation is to preserve tissues permanently in as life-like a state as possible.
4. **Decalcification** - It is the process of removal of the calcium salts from the specimen. This step is required only for tissues containing calcified areas like bone, nail etc. The various agents used for decalcifying are Nitric acid, Hydrochloric acid, Formic acid, Picric acid, Acetic acid, Citric acid.

5. **Bit Taking** : The bits should be of the size of approximately 4- 6 mm in thickness. These bits are then placed in metal cassettes or capsules which are then placed in the fixative. Tiny biopsies or small specimen can be wrapped in a filter paper and then put in a cassette and processed.
6. **Dehydration** : It is the process in which the water content in the tissue to be processed is completely reduced by passing the tissue through increasing concentrations of dehydrating agents.
7. **Clearing** - It is the procedure where in the alcohol in the tissue is replaced by a fluid which will dissolve the wax used for impregnating the tissues
8. **Impregnation**- In this the tissue is kept in a wax bath containing molten paraffin wax for 6 - 8 hours. The wax is infiltrated in the interiors' of the tissue which increases the optical differentiation & hardens the tissue & helps in easy sectioning of the tissue.
9. **Embedding** - It is done by transferring the tissue which has been cleared of the alcohol to a mould filled with molten wax & is allowed to cool & solidify. After solidification, a wax block is obtained which is then sectioned to obtain ribbons.
10. **Section cutting** - It is the procedure in which the blocks which have been prepared are cut or sectioned and thin strips of varying thickness are prepared. The instrument by which this is done is called as a Microtome
11. **Staining** - Staining of the section is done to bring out the particular details in the tissue under study. The most commonly used stain in routine practice is Haematoxylin & Eosin stain
12. **Mounting** - To preserve and support a stained section for light microscopy, it is mounted on a clear glass slide, and covered with a thin glass coverslip. The slide and coverslip must be free of optical distortions, to avoid viewing artifacts. A mounting medium is used to adhere the coverslip to the slide. Aqueous based mounting media are available, which allow the mounting of tissues directly from the staining procedure.

### Fixation

This is the process by which the constituents of cells and tissue are fixed in a physical and chemical state so that they will withstand subsequent treatment with various reagents with minimum loss of architecture. This is achieved by exposing the tissue to chemical compounds, call fixatives. Most fixatives act by denaturing or precipitating

proteins which then form a sponge or meshwork, tending to hold the other constituents. The fixative should be 15 - 20 times more in volume than the specimen

### **Properties of an Ideal Fixative**

- It must be cheap and easily available
- Prevents autolysis and bacterial decomposition.
- Penetrate the tissue and cells rapidly and evenly.
- Preserves tissue in their natural state and fix all chemical components. (proteins, carbohydrates, fats etc.)
- Avoid excessive hardness of tissue.
- Allows enhanced staining of tissue.
- Should be non-toxic and non-allergic for user.
- Providing iso-osmotic conditions to the tissues.

### **Formalin**

Commonly used fixative is 10% formalin. The standard solution is 10% neutral buffered formalin. Buffering prevents any acidity that would promote autolysis and cause precipitation of formol-heme pigment in the tissues. It penetrates the tissue well and fixes the tissue by forming cross-linkages between amino acid residues.

## **4.2.2 Processing of fixed tissue**

In order to cut thin sections of the tissues, it should have suitable hardness and consistency when presented to the knife edge. These properties can be imparted by infiltrating and surrounding the tissue with paraffin wax/low viscosity nitrocellulose / resins or by freezing. This process is called tissue processing. It requires 16-24 hours and done in many stages.

It can be subdivided into

- a) dehydration
- b) clearing
- c) impregnating
- d) embedding.

### **Types of tissue processing**

#### **1. Manual Tissue Processing:**

In this process the tissue is changed from one container of reagent to another by hand.

## 2. Mechanical Tissue Processing

In this the tissue is moved from one jar to another by mechanical device. Timings are controlled by a timer which can be adjusted in respect to hours and minutes. Temperature is maintained around 60°C. (Automated tissue processor) All the before mentioned procedures up to the impregnation step can be done automatically in a single, unmanned instrument, which is the Automated Tissue processor or called Histokinete.



Advantages : It provides constant agitation during every step which ensures better fixation & processing. It reduces the work load & in turns improves the overall output of the laboratory

### Sequence of tissue processing

#### Dehydration:

Tissues are dehydrated by using increasing strength of alcohol; e.g. 50%, 70%, 90% and absolute alcohol. The duration for which tissues are kept in each strength of alcohol depends upon the size of tissue, fixative used and type of tissue. Delicate tissue will get high degree of shrinkage by two great concentration of alcohol. The volume of alcohol should be 50-100 times that of tissue. The various dehydrating agents used are Ethyl alcohol, Acetone, Isopropyl alcohol, Dioxane. Isopropyl alcohol is commonly used.

#### Clearing

During dehydration water in tissue has been replaced by alcohol. The next step alcohol should be replaced by paraffin wax. As paraffin wax is not alcohol soluble, we replace alcohol with a substance in which wax is soluble. This step is call clearing. Clearing of tissue is achieved by any of the following reagents:

- Cedar wood oil : The best agent but is expensive.
  - Benzene : It is carcinogenic.
  - Xylene : It is most commonly used.
  - Chloroform : Toxic and expensive.
- Others are Carbon tetrachloride and Toluene

#### Impregnation with Wax

This is allowed to occur at melting point temperature of paraffin wax, which is 54-60 °C. Volume of wax should be about 25-30 times the volume of tissues. The

duration of impregnation depends on size and types of tissues and the clearing agents employed. Longer periods are required for larger pieces and also for harder tissue like bones and skin as compared to liver kidney, spleen, lung etc

Types of Wax employed for Impregnation:

1. Paraffin wax
2. Water soluble wax
3. Other material, like colloidin, gelatin, paraplast etc.

Paraffin wax is used routinely. It has hard consistency, so section of 3-4 micron thickness can be cut.

### **Embedding**

Impregnated tissues are placed in a mould with their labels and then fresh melted wax is poured in it and allowed to settle and solidify. It is done by transferring the tissue which has been cleared of the alcohol to a mould filled with molten wax & is allowed to cool & solidify. Once the block has cooled sufficiently to form a surface skin it should be immersed in cold water to cool it rapidly. After the block has completely cooled it is cut into individual blocks and each is trimmed. Labels are made to adhere on the surface of the block by melting the wax with a metal strips sufficiently warmed.

### **Types of Moulds:**

- A. Leuckhart's Moulds: are L- shaped brass pieces placed in opposite positions & can be manipulated to increase or decrease the size of the block to be prepared.
- B. Glass or Metal petri dishes :
- C. Watch glass
- D. Paper boats
- E. Tissue -Tek systems

### **Microtomy (Section Cutting)**

Microtome : These are mechanical devices for cutting uniform sections of tissue of appropriate thickness

Types of microtomes:

The Sliding Microtome

The Rocking Microtome

The Sledge Microtome



## Rotary Microtome

### The Freezing Microtome

## Rotary Microtome

Parts of a Microtome (Rotary): Block holder, Knife clamp screws, Knife clamps, Block adjustment

Thickness gauge. Angle of tilt adjustment, operating handle

Rotary Microtome is the most commonly used. Also known as Minnot's Rotary microtome. In this the Block holder moves up and down while the knife remains fixed. The section cutting is effected by the vertical rise and fall of the object against an fixed knife edge. The block holder is equipped with adjustable screws. The block is parallel to the microtome knife. The knife holder is movable. It is suitable for cutting of small tissues & serial sections can be taken on it. For light microscopy, a knife mounted in a microtome is used to cut 4-6  $\mu\text{m}$ -thick tissue sections which are mounted on a glass microscope slide.

Microtome Knife: The Uniform thin serial sections are obtained only with the help of a sharp and good knife. There are different types of knives available like wedge, biconcave etc. Disposable blades are used commonly now a day.

**Tissue floatation bath**-The cut sections are floated on a warm water bath to remove the wrinkles. Then they are picked up on a glass microscopic slide. It is a thermostatically controlled water bath with the inside coloured black. It is maintained at a temperature maintained 5 - 6 degree below the melting point of paraffin wax

### **Section Adhesives**

It is used for fixing the sections on the slides . Adhesives used are Albumin solution ( Mayor's egg albumin), Starch paste and Gelatin

### **Frozen Sections**

Frozen sections are used for rapid diagnosis. Tissue is snap frozen in a fluid and sectioned by a freezing microtome sections are picked up with a glass slide and stained.

### **Cryostat**

Frozen tissue embedded in a freezing medium is cut on a microtome in a cooled machine called a cryostat.

Baking- The slides with the cut tissues are placed in an oven. Baking removes the water between the section and the slide enables proper adherence of the tissue on the slide

## **Staining**

Staining is a process by which we give colour to a section. There are hundreds of stains available.

Acid dye stains basic components e.g. eosin stains cytoplasm. The color imparted is shade of red.

Basic dye stain acidic components e.g. basic fuchsin stains nucleus. The color imparted is shade of blue.

Neutral stains-When an acid dye is combined with a basic dye a neutral dye is formed. As it contains both colored radicals, it gives different colors to cytoplasm and nucleus simultaneously.

Special stains-are used to stain specific components of tissue e.g. fibrous tissue, elastic tissue and nuclear material

Haematoxylin& Eosin stain is the Commonest stain used in Histopathology

## **Mounting**

Mounting Protects and preserves the stained tissue section. It provides better optical quality for viewing under microscope.

DPX ( DistreneDibutyl phthalate Xylene ) is the common Mountant used

## **Filing And Storage of Tissue blocks / Tissue slides**

Processed tissue blocks and examined tissue slides are not disposed immediately but are filed according to their registration number in the tissue block filing cabinet and slide filing cabinet respectively for a minimum period of 5 years for future reference.

### **4.2.3. Diagnostic Cytology**

#### **Introduction**

Cytology is the study of cells. Cytopathology refers to diagnostic techniques that are used to examine cells from various body sites to determine the cause or nature of disease. Father of cytopathology is Dr George Papanicolaou.

#### **Advantages of Cytopathology over Hitopathology-**

Samples can be collected easily and quickly prepared, stained and interpreted.

Inexpensive and little or no risk to the patient

Exfoliative cytology plays a prominent role in the mass screening and the early detection cancer.

Cytological examinations identify disease process (neoplasia vs inflammation, specific vs nonspecific) .

**Disadvantage** may be that some tumors do not exfoliate cells well and therefore may not provide adequate sample to examine. it is not always possible to localize neoplastic lesion

### **Different branches of Cytopathology**

1. Exfoliative cytology -spontaneously shed cells in body fluids
2. Abrasive cytology -dislodges cells from body surfaces. Imprint Scraping, Endoscopic brushing of mucosal surfaces or Washing (lavage) of mucosal or serosal surfaces , Swab
3. Fine needle aspiration cytology-, FNAC

### **Type of specimens**

Urine, CSF, Sputum, Effusions in body cavities like pleura, Cervical smear, buccal scrapings Imprint Scraping, Endoscopic brushing of mucosal surfaces or Washing (Lavage) of mucosal or serosal surfaces, Swabs, needle aspirates

### **Preparation of smear**

Fresh specimen is always recommended for cytological examination .Smears are prepared from the specimen and are fixed immediately. Fluid specimens are centrifuged and the smear is prepared from the deposit .

### **Fixation**

Cytological specimens / smears are immediately fixed to avoid morphological changes of cells. The classic cytological fixative is Ether- Ethyl alcohol mixture. Carnoys fluid, 95% ethyl alcohol, isopropyl alcohols are other fixatives used in cytology.

### **Staining technique**

Staining techniques employed mainly are

- Papanicolaou staining
- Shorr's staining for assessment of hormonal status and demonstration of barr body

The most commonly employed cytological staining technique is Papanicolaou staining (PAP staining)

### **Applications of Diagnostic cytology**

1. Diagnosis of cancer

2. Assessment of hormonal status
3. Diagnosis of infections
4. Sex determination ( Barr body)

### DETAILING OF PRACTICALS

**Demonstrations of Tissue block and stained H&E histology sections.**

### ASSESSMENT ACTIVITIES

Chart preparation on different steps in tissue processing

Assignment on common specimens used in cytology

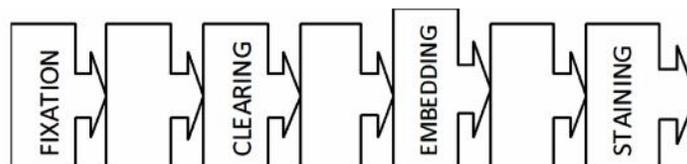
### Theory Evaluation Questions

- 1 Complete the given table with suitable words

Commonly Used	Name
Fixative	
	DPX
Microtome	
	Chloroform

2

- 2 a) Complete the flowchart showing the different steps in processing of a tissue for microscopic examination. (3 score)



- b) Explain the fixative and stain commonly used in Histopathology (2+2)
- 3 Prepare a flow chart showing the different steps undertaken for processing a tissue specimen in a histopathology laboratory. (5)
- 4 Histokinete and Cryostat are two equipments used in histopathology laboratory. Differentiate between the two. (4)

5. A group of VHSE students are coming for a field visit to your histopathology lab. Give description about different steps of tissue processing in histopathology. (4)
6. Write down the name of a reagent used for decalcification in a histopathology lab (1)
7. Differentiate the followings (4)
  - A) Histology-Cytology
  - B) Biopsy-autopsy
  - C) Explain the advantages of Histokinetic in tissue processing (3)
8. Give reasons
  - i) 10% Formalin is an ideal fixative for tissue fixation (2)
  - ii) Rotary microtome is popular in a histology lab than any other microtome (3)
9. FNAC is quite common now. Explain 3 advantages of FNAC over a tissue biopsy (3)
10. Most routinely used stain is ----- in histopathology lab and ----- is in cytology lab (2)

### EXTENDED ACTIVITIES

- Organize a visit to Microbiology & Histopathology depts. of a Laboratory/ Medical College to observe Microbial identification procedures and Histo & Cytotechniques.
- To conduct a Survey on various vector borne diseases.
- Participate in the various programs such as AIDS awareness , Filariasis eradication, health campaigns etc conducted by the health dept or NGOs.
- Exhibition/Expo

### List of Practicals

1. Demonstration of operation procedure of hot air oven
2. Demonstration of operation procedure of Autoclave
3. Preparation of liquid media - Peptone water

4. Preparation of solid media - NA, BA, CA, MA
5. Handling, sterilisation and use of bacterial wire loop
6. Demonstration of inoculation methods
7. Inoculation of Culture media- Liquid
8. Perform hanging drop method of motility
9. Inoculation of Culture media- Solid
10. Demonstration of colony characters of commonest pathogens
11. Preparation of bacterial smear- from liquid media
12. Preparation of bacterial smear- from solid media
13. Perform simple staining
14. Perform Gram staining
15. Perform AFB staining
16. Demonstration of ABST
17. Model/Chart preparation of a typical immunoglobulin
18. Demonstrate and Perform RPR Test
19. Staining and examination of thin smear for blood parasites
20. De-haemoglobinisation, Staining and examination of thick smear for blood parasites
21. Demonstration of wet smear for filarial parasite
22. Perform direct smear of stool sample for parasites-saline and iodine
23. Demonstration of floatation/sedimentation Techniques for intestinal parasites
24. Demonstrations of Tissue block and stained H&E histology sections.

### List of References

Recommended Text Books

#### Module 3

1. Text book of Medical Laboratory Technology - RamnikSood
2. Manual of Laboratory safety - RoshidNajat
3. Practical Clinical Biochemistry methods and interpretations- Ranjana Chawla
4. Laboratory procedures in haematology -Mehdi SR
5. Essentials of Blood banking -Mehdi SR
6. The short text book of Medical Laboratory for technicians- Satish Gupte
7. A hand book of Clinical Pathology- GanendrakumarChakravarti

8. Medical Laboratory science: Theory and practice- J Ochei. A.Kolhatkar
9. ParcticalHaematology- Dacie and Lewis
10. Text book of Medical Laboratory Technology Vol I & II - PrafulGodkar, DarsanGodkar
11. MedicalLvaboratory Technology , Procedure manual for Routine diagnostics Vol I, II & III - Kanai Mukherjee
12. Lynch,s Medical Laboratory Technology- Stanley S Raphael, Mathew J Lynch.
13. Medical laboratory mnnual for Tropical countries vol I & II Monica Cheesbrough
14. Basic Medical Laboratory Techniques Barbara H.Estridge et al
15. Tietz-fundamentals ofclinicalchemistry

**Reference Sites:**

Web sites <https://en.wikipedia.org>,  
<https://internalmedicine.osu.edu>,  
<https://labtestsonline.org>  
<https://www.dcccd.edu>,  
<https://www.nlm.nih.gov>,  
[www.science.edu](http://www.science.edu),  
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[www.sciencedirect.com](http://www.sciencedirect.com),  
[www.encyclopedia.com](http://www.encyclopedia.com),  
[www.healthline.com](http://www.healthline.com), [www.webmed.com](http://www.webmed.com),  
[www.acb.org](http://www.acb.org)

**Module 4**

1. Prescott's Microbiology, 9th Edition  
Authors: Joanne Willey, Linda Sherwood and Christopher J. Woolverton
2. JawetzMelnick&Adelbergs Medical Microbiology, 26th Edition  
Authors: Geo. Brooks, Karen C. Carroll, Janet Butel and Stephen Morse
3. Bailey & Scott's Diagnostic Microbiology, 13th Edition  
Author: Patricia Tille
4. Greenwood Medical Microbiology, 18th Edition  
Authors: David Greenwood, Richard C. B. Slack, Michael R. Barer and Will L Irving

5. District Laboratory Practice in Tropical Countries, Part 1&2, 2nd Edition  
Author: Monica Cheesbrough
6. Medical Microbiology, 7th Edition  
Authors: Patrick R. Murray, Ken S. Rosenthal and Michael A. Pfaller
7. Lippincott's Illustrated Reviews: Microbiology, 3rd Edition  
Authors: Richard A. Harvey and Cynthia Nau Cornelissen
8. Microbiology: An Application Based Approach  
Author: Michael J. Pelczar, ECS Chan and Noel R. Krieg
9. Koneman's Color Atlas and Textbook of Diagnostic Microbiology, 6th Edition  
Author: Elmer W. Koneman
10. Foundations in Microbiology, 8th Edition  
Authors: Kathleen Park Talaro and Barry Chess
11. Textbook of Diagnostic Microbiology  
Authors: Connie R. Mahon, Donald C. Lehman, George Manuselis
12. Medical Microbiology, 3rd Edition  
Authors : Cedric Mims , Hazel Dockrell, Richard Goering
13. Ananthanarayan and Paniker's Textbook of Microbiology, 9th Edition ArtiKapil
14. Medical microbiology Prep manual for undergraduates, 2nd Edition  
Author: Asha P Pichare, B. S Nagoba
15. Textbook of Microbiology, 4th Edition Author -Baveja
16. Basic laboratory procedures in clinical bacteriology 2nd Edition World health organization
17. Parasitology 13th edition K.D. Chatterjee.
18. Paniker's Textbook of Medical Parasitology
19. WHO- Basic Laboratory Methods in Parasitology

#### Reference sites

<http://www.asm.org/American society for microbiology>

<http://www.cdc.gov/Centers for Disease control>

<http://www.nih.gov/National institute of health>

<http://www.who.int/home-page/WHO>