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# **Comprehensive Manual of Clinical Microbiology for Postgraduates**

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**Comprehensive Manual of Clinical Microbiology for  
Postgraduates**

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## **Preface**

**The revised edition has been done with the revision of bacteriology, serology, and infection control. We have revised questions from certain books.**

This manual has been written with the intention of guiding postgraduate students entering the field of microbiology. This book will help the PG students to navigate the new curriculum and is designed to further their knowledge in the field. Microbiology has always been a worrisome subject for most, studied mainly with the intent of clearing the UG (undergraduate) examinations. However, the author, a microbiologist and medical practitioner for many years attests that good microbial knowledge can be a valuable aid in clinical practice and guiding physicians. This book guides the students towards resources needed to enhance their knowledge in the field of microbiology and stressed the importance of documentation. The field of Microbiology has undergone tremendous changes over the past few years, especially in the wake of the COVID-19 pandemic.

With tests like RT PCR (Reverse transcription polymerase chain reaction) being the norm, even the public is more aware of microbiology and its importance. As clinical microbiologists, the students should have good research knowledge and analytical skills and must work closely with physicians and not limit themselves to the lab alone.

This manual may not be in its complete form and may require some new updates of the contents whenever syllabus is revised.

**The procedures and methods followed in the exercises are widely varied and the students are advised to adhere to the center's own policies.**

**The questions and answers discussed in this manual are based on the standard books and own views. It may be used under one's own risk.**

## Foreword



The author of this Practical Manual of Clinical Microbiology for Postgraduates, Dr E Subbalakshmi is known to me for the last decade. The manual has all the information needed by a postgraduate in Medical Microbiology with emphasis on the basic tests which need clear understanding by the students. The author has done a great work in this regard by referring to standard books and giving a clear explanation for majority of tests done for identification of common pathogens.

The manual is in concurrence with NMC prescribed CBME curriculum for post graduates in MD Microbiology. In this era of pandemic induced rise in infections of hospital acquired bacterial pathogens, a postgraduate must be in a position for a quick interpretation of tests for identification of the isolate and guidance in treatment to the clinicians. This manual will serve the purpose to a large extent. The author needs a lot of appreciation in this regard.

I sincerely congratulate the author, Dr E Subbalakshmi for her efforts in bringing out this much needed practical manual for postgraduates in Medical Microbiology and my best wishes for its success in the medical community.

Dr Girish N

Professor of Microbiology

Vydehi Institute of Medical Sciences and Research Centre, Bangalore



The author, Dr. E. Subbalakshmi, is an MBBS, MD Microbiologist, working as an Associate professor in the Department of Microbiology, Saveetha Institute of Medical and Technical Sciences, Chennai. The author was inspired by her father, Late Mr. S. Easwaran, a schoolteacher who always helped students and encouraged them in their studies. The author would like to give a special mention to her mother, versatile woman who pursued her passion till the end.

The author would like to give a special mention to Professor Dr. S. Jayakumar, currently working at, Saveetha Institute of Medical and Technical Sciences, Chennai and Professor Dr.V. Kalpana Devi, currently working at ACS Medical College and Hospital, Chennai. They were instrumental at each step in guiding the author with their valuable knowledge and inputs. The author would also like to thank her mentor, Dr.Balasubramanian, ENT Surgeon, Dean, Lalithambigai Hospital, Chennai, and her post graduate students. The author would finally like to thank Dr. Deepak, Director of Saveetha Institute of Medical and Technical Sciences, Chennai. and Dr. Neelushree, HOD in charge, Saveetha Institute of Medical and Technical Sciences, Chennai Department of Microbiology, for their valuable support.

The author would like to dedicate this book to her beloved parents and family.

## COAUTHORS



Dr. S. Jayakumar is currently a Professor in the Department of Microbiology, Saveetha Institute of Medical and Technical Sciences, Chennai. He is an inspiring microbiologist who constantly motivates students and colleagues in the field. He has more than 16 years of professional experience and has several national and international publications to his name. He is very passionate about teaching UG and Post graduate students.



Dr. V. Kalpana Devi, currently Professor of Microbiology, Principal, Allied Health Sciences, ACS Medical College and Hospital, Dr. MGR Medical Educational and Research Institute, has 12 years of professional experience in the field of Microbiology and is passionate about virology and immunology. She has several national and international publications and has trained several students in her academic career. Her passion towards writing motivated her to publishing this book for postgraduates.

## Recommended books for reading

1. Bailey & Scott's Diagnostic Microbiology, 14th Edition. Patricia M. Tille
2. Discrete Laboratory Practice in Tropical Countries. Second edition. Part 1 and 2, Monica Cheesebrough
3. Winn, W., Allen, S., Janda, W., Koneman, E., Procop, G., Schreckenberger, P. and Woods, G. (2006) Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th Edition, Lippincott Williams and Wilkins, New York.
4. Ananthanarayan and Paniker's Textbook of Microbiology. Author: Reba Kanungo  
Edition: 10th Edition
5. Essentials of Medical Microbiology--Apurba Shankar Sastry, Sandya Bhat K
6. Paniker's textbook of Medical Parasitology--Jayaram Paniker
7. Essentials of Medical Parasitology. Apurba Sankar Sastry, Sandhya Bhat K.
8. Fungal infections Diagnosis and management--Malcolm. D. Richardson and David W. Warnock
9. Kuby Immunology 7th edition
10. Textbook of Practical Microbiology--Subash Chandra Parija
11. Fields Virology
12. Jawetz, Melnick and Adelberg's Medical Microbiology --25th edition
13. Textbook of Medical Mycology-- 4th Edition Jagadish Chander
14. Mackie and McCartney -----J.G. Collee, A G Fraser, B.P. Maramon, A. Simmons
15. NMC guidelines for postgraduates 2020-21.
16. Essentials of hospital infection control Apurba S. Sastry
17. Essentials of MD/DNB Microbiology Practical examination Dr.C.P.Baveja,Dr.V.Baveja
18. Clinical and Laboratory Standard Institutes -Performance standards for antimicrobial susceptibility tests 13<sup>th</sup> edu.

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## **1.Introduction**

The following subjects are of utmost importance to a Microbiologist and must be covered over the course of 3 years of the Post Graduate Programme: General Bacteriology, Systemic Bacteriology, Virology, Parasitology, Immunology, Mycology and Molecular diagnosis. The student needs to interact with the allied departments and acquire knowledge of infections in humans, immune prophylaxis, transplantation immunology, health care associated infections, antibiotic stewardship, and outbreak investigations. The student needs to be aware of national policies like Revised National Tuberculosis Control Programme (renamed as National Tuberculosis Elimination Programme), NACO (National AIDS Control Programme), etc. In addition to the above, the students need to have knowledge about quality assurance, quality control and management for laboratory standardization and accreditation, which is an essential prerequisite of the curriculum.

## 2. General guidelines

### 2.1. Duties and responsibilities

- As part of your Post Graduate Programme, you will spend the first 3 months in rotation, where you will undergo orientation for all the divisions in Microbiology. There are seven divisions: Bacteriology, Media Preparation, Virology, Immunology, Mycology, Parasitology and *Mycobacterium tuberculosis* learning section and Molecular diagnostics. After the orientation you will be posted in each division in rotation.
- In addition, you will have clinical postings pertaining to Microbiology and also peripheral postings in district headquarters for 3 months.
- At the end of the 3-year course, the student must be able to
- Develop the art of eliciting clinical history from patients to correlate laboratory reports.
- Develop communication skills to inform critical parameters to clinicians without undue delay.
- Diagnose infectious diseases, correlating the clinical details with laboratory tests as evidence.
- Follow moral ethics in maintaining the confidentiality of laboratory reports.
- Notify reports of infectious diseases such as Dengue, Cholera, Malaria etc.
- Identify, aid in prevention, control, and treatment of common Health Care Associated Infections (HCAI) which include Catheter Associated Urinary Tract Infections (CAUTI), Ventilator Associated Pneumonia (VAP), Catheter related Blood Stream Infections (CRBSI), Surgical Site Infections (SSI), Peripheral line Associated Blood Stream Infections (PLABSI).
- Interpret the surveillance indicators of HCAI (Health Care Associated Infections)

- Practice and participate in Antimicrobial Stewardship Program (ASP) with regard to selection of appropriate antimicrobials – dose, duration, dosing interval and de-escalation, etc.
- Alert the treating physician on existing and emerging antimicrobial resistance and follow up.
- Undergo training in research methodology, medical ethics, bioethics and medico-legal aspects.
- Attend workshops and CMEs conducted by the University and other reputed institutions.
- Should complete a research topic during the entire course of 3 years

## 2.2. Safety precautions

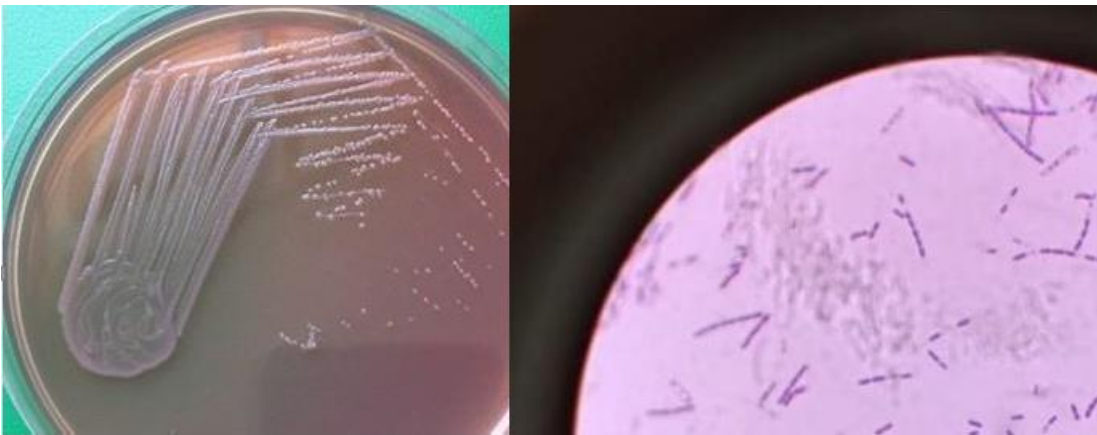
As a microbiologist, safety is always first since you handle dangerous, harmful pathogens. The safety precautions to be followed in lab are as follows:

- (i) Always enter the lab with your apron.
- (ii) Disinfect the working surface before and after the procedure.
- (iii) Wash your hands before and after leaving the lab.
- (iv) Do not carry edibles inside the lab
- (v) Do not eat, drink, smoke or use any sort of cosmetics inside the lab.
- (vi) Do not bite or chew pens. Use water to moisten specimen labels and not the tongue (**this is a common malpractice amongst most technicians**)
- (vii) Do not insert or remove contact lens in the lab.
- (viii) Do not maintain long nails and do not expose your hair outside.
- (ix) Restrict your conversations to a minimum.

- (x) Take precaution to prevent skin and mucous membrane exposure, including always wearing gloves along with masks, goggles, gowns, or aprons, in case splash or droplet formation is a risk.
  
- (xi) Treat all samples as infected, until proven otherwise and observe universal precautions while processing the samples.
  
- (xii) Discard sharp waste in an appropriate, puncture-resistant container.
  
- (xiii) Do not recap needles by hand.
  
- (xiv) Disinfect all instruments immediately after use.
  
- (xv) Disinfect all contaminated waste before discarding in 1% hypochlorite solution (Remember hypochlorite should be always freshly prepared. Should be discarded after 24 hours).
  
- (xvi) Report all accidents or exposures to infectious agents immediately, to the concerned authorities.
  
- (xvii) Vaccinate yourself against Hepatitis B (3 doses 0, 1 and 6 months) and if you have already been vaccinated, make sure your antibody titres are checked. The antibody titre should be  $>10.0$  IU/ml.
  
- (xviii) In addition, I would suggest you take Fluvaccine, Covishield or Covaxin for prevention against corona virus if you are going to work in the RTPCR lab.
  
- (xix) You should also have knowledge about handling of accidental spillage of chemicals, biomedical waste management and about fire safety, electrical safety.

**Spend maximum time in the lab, observe the teachers when they work and learn from their experience.**

### 3. BACTERIOLOGY



When you think of the Microbiology department, you must always be focused on bacteriology, since the skill of the microbiologist is assessed by their ability to interpret the results.

Microbiological investigations are important in the diagnosis, treatment and surveillance of infectious diseases and policies regarding the selection and use of antimicrobial drugs. Hence, the test reports should be reliable, and standardized and should provide the required information in a format that can be understood. The following apply to the Quality Assurance (QA) of the pre-analytical, analytical, and post-analytical stages of microbiological procedures and should be incorporated in microbiological SOP (Standard operating procedure).

### **3.1 Pre analytic stage- Sample collection**

#### **3.1.1. Criteria to be strictly followed**

This phase is very important before you proceed to process the samples. The following should be observed in preanalytical phase.

The requisition form has to be filled correctly with the following:

Details of the patient like name, age, sex, occupation and residential address must be filled properly. Clinical diagnosis, history of treatment with any drugs, name of the medical officer requesting for the investigation.

History of drug allergy

Make sure the correct specimen has reached the lab, and at the right time.

The material for collection should be from the actual site of infection, collected with minimum contamination from adjacent tissues, organs, or secretions.

Optimal times for collection of specimens must be established to provide the best chance of recovering causative organisms.

Enough specimen must be obtained to perform the requested tests

Appropriate collection devices, specimen containers and culture media must be used to ensure optimal recovery of microorganisms.

#### **Note:**

Eg. Samples that require immediate attention: C.S.F, blood cultures, unpreserved urine, swabs not transported in transport media, faecal specimens containing blood and mucus and wet slide preparations.

### 3.1.2 Instructions for sample collection

**Urine** - Clean catch mid-stream urine collection (boric acid is used as preservative if the sample is not processed immediately)

**Sputum** - Need to cough and bring out the sputum in a wide mouth sterile container.

**Throat swab** - Should be taken with the mouth wide open from both the tonsillar area. Make sure the patient has not used any antiseptic solution for gargling.

**Blood culture** - 10 to 20ml of blood should be collected and half of the sample should be inoculated into each of a "set" of two culture bottles (both for aerobic and anaerobic) which may contain different media and incubated in different atmospheres. 2.5 to 5ml required in paediatric patients (**sodium polyenathol sulfonate 0.25% used as preservative**)

**Pus** from any site - Should be taken with the sterile swab and should not touch the skin of the patient.

**Stool** - Wide mouth container (polyvinyl alcohol and buffered formalin used as preservative).

### 3.1.3 Criteria for sample rejection

Samples which should not be accepted for microbiological investigations include.

**Dry fecal swab**

**Requisition form that does not match with the collected sample**

**Saliva instead of sputum and twenty-four-hour sputum collection.**

**A single swab submitted for multiple requests eg. aerobes, anaerobes, fungus, and tuberculosis.**

**Eye swabs not freshly collected.**

**Leaking specimen**

**Any specimen not collected into a correct container.**

**Any specimen received in formalin.**

## 3.2. Analytic stage

### 3.2.1. Criteria to be followed in processing of the samples.

Sample processing plays an important role in bacteriology. Any sample received should be processed within 2 hours. If there is a delay in processing, the procedures given for

the preservation of samples must be followed. Serum for serological studies must be stored at -20 deg for 1 week. Tissue samples can be stored at -70 deg for 1 year. Samples which harbour delicate pathogens need to be transported in transport medium. For example, in case of *Vibrio cholerae* the sample should be transported in Venkatraman Ramakrishnan medium.

If there is a delay in processing you need to refrigerate the sample. **CSF and blood culture samples should not be refrigerated.** They are kept in incubator. As soon as the sample is received check the sample with requisition forms.

### **3.2.2 Quality check to be done regularly**

Before processing the sample, make sure the following items are maintained in the lab. All stains and reagents should be clearly labelled, dated, and stored correctly. Stains and reagents should not be used beyond expiry dates. There should be documents of daily maintenance of temperature of incubator, hot air oven, refrigerator, water bath.

Quality control of stains for gram stain and acid-fast stain to be done every day before testing the samples. Quality control of culture plates and antibiotic sensitivity of the discs should be maintained regularly with ATCC (American Type Culture Collection) strains: *Staphylococcus aureus* 25923, *Escherichia coli* 25922, *Pseudomonas aeruginosa* 27853 and *Enterococcus faecalis* 29212. Sterility checks of the culture plates to be done regularly before processing with positive and negative control strains for the corresponding plates. SOP (Standard operating procedure) and QC (Quality control) of equipments used in the microbiology laboratory should be maintained, eg. microscope, incubator, anaerobic jar, centrifuge, water bath, autoclave, hot-air oven and refrigerator. For each item of equipment there should be clear operating and cleaning instructions and service sheets should be maintained. Regular cleaning, servicing and maintenance are essential if equipment must remain safe to use and in good working condition. **Internal quality control should be done initially for antibiotic susceptibility testing consecutively for 20 days and then weekly once if the initial results are concordant.**

### **3.2.3. Microscopic examination before processing the samples**

All specimens should be subjected to direct microscopic examination. This helps in several purposes before the sample is processed.

1. The quality of the specimen can be assessed.

2. The microbiologist can help the physician with the gram stain report at the earliest.
3. The work up of the sample can be guided by what is growing on culture and what is observed in direct smear.

**Note:** Direct smears are not performed for stool samples.

### **3.2.4 Critical samples that require immediate attention.**

- Positive blood culture
- Positive gram stain of CSF
- Smear suspected of Malaria.
- Smear suspected of gas gangrene.
- Stool sample suspected of *Vibrio cholerae*
- Smear suspected of *Streptococcus spp.* in surgical wound.

### **3.3 Macroscopic examination of samples**

Macroscopic examination of samples is important as it helps to clinically identify some pathogens.

**Pus**— Generally received in swab will be difficult to see macroscopically. However, look for the colour of pus which varies from green, yellow to brown red. A red colour is generally due to mixture with blood or haemoglobin.

Pus from postoperative or traumatic wounds (burns) may be stained blue green by the pyocyanin pigment produced by *Pseudomonas aeruginosa*. The aspirate from a primary amoebic liver abscess has a gelatinous consistency and a dark brown to yellowish brown colour.

**Consistency:** The consistency of pus may vary from a turbid liquid to one that is very thick and sticky. Exudates aspirated from a joint, the pleural cavity, the pericardial sac, or the peritoneal cavity are generally liquid. Pus originating from a draining sinus tract in the neck should be inspected for small yellow “sulfur” granules, which are colonies of the filamentous *Actinomyces israelii*.

Small granules of different colours (white, black, red, or brown) are typical of mycetoma, a granulomatous tumour, generally involving the lower extremities (e.g., madura foot), and characterized by multiple abscesses and draining sinuses. The coloured granules correspond to either filamentous bacteria or fungal mycelium. Pus from tuberculous “cold abscesses” (with few signs of inflammation) is sometimes compared with soft cheese and called as caseous pus.

**Odour:** A foul odour is one of the most characteristic features of an anaerobic or a mixed aerobic - anaerobic infection, although it may be lacking in some instances. The odour together with the result of the Gram-stained smear, should be reported to the clinician as early as possible as it may be helpful in the empirical selection of an appropriate antibiotic.

**Note:** For anaerobic culture, it should be collected in syringe with needle, the needle should be closed and should be plunged into a cork bottle to prevent entry of oxygen.

**Urine**---Look for colour

Cloudy urine—urinary tract infection

Yellowish brown urine—Hepatocellular jaundice.

Red and cloudy urine—Urinary schistosomiasis, bacterial infection.

Milky white chylous urine—*Bancroftian filariasis*

**Sputum**—Colour, consistency—Mucoid, purulent, mucopurulent, presence of blood.

A sample of sputum is considered unsuitable when the sputum is mostly saliva and when the sample has large numbers of squamous epithelial cells and only a few or no pus or macrophage cells. This can be assessed by

### **Barlet grading of sputum samples**

#### **No. of neutrophils per 10X low- power field**

**<10 grade 0**

**10-25 grade +1**

**>25 +2**

#### **Presence of mucus +1**

#### **No of epithelial cells per 10X low -power field**

**10-25 grade -1**

**>25 grade -2**

Average the number of epithelial cells and neutrophils in about 20 or 30 separate 10 X microscopic fields and then calculate the total.

A final score of 0 or less indicates the lack of active inflammation or contamination with saliva.

**Stool**—colour, consistency, blood, volume

## Effusions

Describe colour and whether:

Clear, cloudy, or purulent

Blood stained

Contains clots (sample without anti- coagulant)

Contains pus or blood stained.

### Samples appearance in effusion

	Transudate	Exudate
Appearance	Clear pale yellow	Purulent, cloudy or blood stained
Clotting	Does not clot	Often clots
Cells	Few cells	Purulent, cells, mostly neutrophils Non-purulent, few and many cells; mostly lymphocytes
Protein	Less than 30g/dl	More than 30g/dl

## 3.4. Processing of sample

### 3.4.1. Processing of samples on the plating media

According to the sample, we need to do direct smear for all the samples except stool and blood. The samples should be streaked in corresponding plates. The plating media depends on the sample to be processed.

Sputum—Blood agar, Chocolate agar, Macconkey agar with crystal violet

Throat swab—Blood agar, Chocolate agar, Mac conkey agar with crystal violet

Urine—Blood agar, Mac conkey agar, CLED agar (Cysteine Lactose Electrolyte Deficient Agar), Hichrome UTI agar.

Stool- XLD (Xylose lysine deoxycholate citrate agar), DCA (Deoxycholate Citrate Agar), Mac conkey agar, Selenite F broth. (Details about processing of stool sample including hanging drop and culture will be discussed in parasitology section 5.7)

Sterile fluids—Blood agar, Chocolate agar, Mac conkey agar

Before you process the sample, the plates required for streaking should be dried properly otherwise water condensation can result in contamination of the plates.

### **3.4.2. Procedure to make direct smear**

Take a clean slide and label it.

Every slide must be labeled clearly with the date, the patient's name, and lab identity number. Whenever possible, smears should be spread on slides which have one end frosted for labeling. The smears should be approximately 15X20mm.

### **3.4.3. The techniques used to make smears from different specimens**

**Purulent specimen:** Using a sterile wire loop, make a thin preparation. Do not centrifuge a purulent fluid, e.g., C.S.F. containing pus cells.

**Non-purulent fluid specimen:** Centrifuge the fluid and make a smear from a drop of the well-mixed sediment.

**Sputum:** Use a piece of clean stick to transfer and spread purulent and caseous material on a slide. Soak the stick in phenol or hypochlorite disinfectant before discarding it.

**Swabs:** Roll the swab on a slide. This is particularly important when looking for intracellular bacteria such as *N. gonorrhoeae* (urethral, cervical, or eye swab). Rolling the swab avoids damaging the pus cells.

**Faeces:** Use a piece of clean stick to transfer pus and mucus to a slide. Decontaminate the stick before discarding it. Spread to make a thin preparation.

### **3.4.4. Methods of fixation of smears**

There are three methods of fixation of smears

#### **Heat fixation**

**Alcohol fixation (95 % methanol for 1min)**

#### **Chemical fixation**

Purpose of fixation is to prevent smears being washed away while staining and also to inactivate the bacteria.

**Note:** When doing heat fixation make sure you don't overheat it. This can be assessed by gently passing the slide over the flames and keeping the slide on the dorsal part of your hand, the heat should be bearable.

Alcohol fixation is done for organisms which are intra cellular. Chemical fixation is done using 40g/l potassium permanganate.

Use a staining rack. Before you proceed for stain, check whether all the stains are present. Use a dropper to pour the stains.

### **3.4.5 Precautions to be taken while staining the smears**

First check the thickness of the smear. You can assess the thickness by checking with the newspaper. If you are able to read the paper, the thickness is perfect.

Too thick and too thin smear will give false positive result. Follow the steps of staining procedure as given in the kit. Too thick and too thin smear will give false positive result. Follow the steps of staining procedure as given in the kit.

The important step is decolorizing.

### **3.5. Gram stain**

Gram stain—Generally gram stain is done for all bacterial organisms. Gram stain is the most important staining method in bacteriology. It is the only method employed for the diagnostic identification of bacteria in clinical specimens. Beside demonstrating the morphology of bacteria, it distinguishes between genera. Gram positive which stain dark purple, and gram-negative which stain light red.

Steps of gram stain.

Step 1—Crystal violet for 1min. Wash with water.

Step 2—Gram's iodine for 1min. Wash with water.

Step 3—Decolorizing agent acetone for 2 sec. Wash with water.

Step 4—Counter stain with safranin for 1min. Wash with water.

After final wash, air dry the slide and use oil immersion objective to view the slide.

In each hospital, the primary stain, decolorizer, and the counter stain can vary.

Follow the kit instructions.

Gram stain was invented by Hans Christian Gram who was a histologist. It is used to identify pathogen and organisms in the specimen.

## Questions

1. What type of stain is Gram stain?

It is a differential stain which is used to differentiate between Gram positive and Gram-negative bacteria.

2. Who invented Gram stain?

Gram stain was invented by Hans Christian Gram who is a histologist.

3. Mention the different steps of Gram stain.

Steps of gram stain.

Step 1—Crystal violet for 1min. Wash with water.

Step 2—Gram's iodine for 1min. Wash with water.

Step 3—Decolorizing agent acetone for 2 sec. Wash with water.

Step 4—Counter stain with safranin for 1min. Wash with water.

4. Mention the different types of primary stain.

The Primary stains can be Crystal violet, Gentian violet, Methyl violet.

5. Mention the different counter stains that can be used?

The different counter stains include Neutral red (1%), Safranin (0.5%) and Dilute carbol fuchsin and Basic fuchsin.

Neutral red for 2-4min, demonstrating *Gonococci* and other intracellular gram-

negative bacteria.

Safranin and dilute carbol fuchsin for 10-30 seconds.

Basic fuchsin for 10-30 seconds.

6. How do you prepare dilute carbol fuchsin?

Dilute carbol fuchsin is prepared by 1:10 dilution of concentrated carbol fuchsin.

7. Mention the different decolorizers you know?

Different decolorizers are absolute alcohol, acetone, iodine acetone, acetone-alcohol.

8. Mention the time duration for each decolorizer.

Acetone: 2- 3sec

Acetone-alcohol: 10 sec

Iodine- acetone: 2 sec to 30 sec

Absolute alcohol: 1min

9. What is the theory of Gram stain?

There are 2 theories.

**pH theory.** Cytoplasm of the Gram positive bacteria is more acidic and retains the basic dye ie crystal violet for a longer time. Iodine which is a mordant form a complex with the dye and gets retained in the cell.

**Cell wall theory.** Gram positive bacteria have thick peptidoglycan layer with tight cross linkage .This acts as a permeability barrier preventing the loss of crystal violet. The dyeiodine complex is formed with the help of mordant in the cytoplasm. Moreover at the time of decolorisation the pores of peptidoglycan shrink which helps in retaining the crystal violet. Besides the lipid content is less which leads to formation of small pores and prevent escape of dye from the cytoplasm. In case of gram-negative bacteria, they have thin peptidoglycan layer which is not tightly cross linked and is more permeable to the dye. In addition the presence of lipopolysaccharide present in gram negative bacterial cell wall gets disrupted with acetone which result in formation of large pores during decolorisation. So at the time of decolorisation the dye comes out of the cytoplasm and the smear takes the pink colour.

10. What is the content of Gram's iodine and Lugol's iodine?

Lugol's iodine was used by Hans Christian Gram. It contains Iodine 1g, Potassium Iodide 2g, water 300ml.

Gram's iodine contains Iodine 10g, Potassium iodide 20g, Distilled water 1000ml.

11. Which stain was used by Gram?

He used Aniline- Gentian violet as primary stain, Lugol's iodine as mordant and absolute alcohol as decolorizer. The counter stain was bismarck brown.

12. What is the positive control and negative control used for Gram stain?

Positive control *S. aureus* ATCC 25923

Negative control *E. coli* ATCC 25922

13. What is ATCC?

American Type Culture Collection.

14. What is NCTC?

National Collection of Type Cultures.

15. What is MTCC?

Microbial Type Culture Collection and Gene bank.

16. What are all the different modification of Gram stain?

**Kopeloff and Beerman's**-- Methyl violet for 5min, Gram's iodine for 2 min, decolorizer is acetone and counter stain basic fuchsin for 30 sec.

**Jensen's method**—Methyl violet for 30 sec, Gram's iodine for 30 sec, decolorizer absolute alcohol for 30 sec, neutral red (0.1%) for 2min.

**Preston and Morolle's**—Crystal violet for 30 sec, Gram's iodine for 30 sec, Iodine acetone for 30 sec, dilute carbol fuchsin for 30 sec.

**Quick gram stain**—Crystal violet for 5 sec, Gram's iodine for 5 sec, acetone, Basic fuchsin for 5 sec.

**Hucker's modification**--Crystal violet for 30sec, Gram's iodine for 30 sec, Acetone alcohol decolorizer for 10 sec, Safranin for 30 sec.

17. Mention the different types of stains.

Simple stain-Methyl violet, Dilute carbol fuchsin.

Differential stain- Gram stain, Ziehl -Neelsen stain, Albert stain

Negative stain—India ink, Nigrosin stain for demonstration of capsule

Silver impregnation stain, Fontana stain for demonstration of spirochaetes

Ryu's stain for flagella

Malachite green stain, modified Ziehl-Neelsen stain (0.25%) sulphuric acid as decolorizer for spores

Periodic acid -Schiff (PAS) stain for demonstration of tissues for histopathology slides.

Fluorescent stains (Acridine orange, Auramine rhodamine)

Romanowsky stain for malaria (impart a reddish-purple colour to the chromatin of malaria and other parasites).

Leishman's stain, Giemsa stain for protozoa

Levaditi stain for staining of spirochaetes in tissues

18. What modification of Gram's stain is used in your laboratory?

We are following Hucker's modification for Gram stain. (This is followed in our lab. You need to frame it according to modification followed in your lab).

19. What is gram variability?

Gram positive organisms may lose their ability to retain crystal violet and stain Gram negatively for the following reasons:

- Cell wall damage due to antibiotic therapy or excessive heat-fixation of the smear, over-decolorization of the smear.
- Use of an iodine solution which is too old, i.e., yellow instead of brown in colour (always store in a brown glass or other light opaque container).
- Smear has been prepared from an old culture.
- Gram negative organisms may not be fully decolorized and appear as Gram positive when a smear is too thick.

20. When do you use only dilute carbol fuchsin to stain?

The use of dilute carbol fuchsin (1 in 10) is recommended for staining *Vincenti organisms*, *Yersinia*,

*Haemophilus*, *Campylobacter* and *Vibrio* species.

21. How do you confirm gram stain reaction by KOH?

The purpose of KOH is to identify Gram negative bacteria. In the presence of KOH, the cell wall of gram-negative bacteria are destroyed as they dissolve thin layer of peptidoglycan layer of gram negative cell wall easily.

22. What are the different methods of fixation of smears?

23. How do you assess thickness of the smear?

24. What is the size of the smear?

Answers for 22, 23 and 24 are explained above in analytical phase.

25. How do you process the samples?

Answer explained above in analytical phase.

26. How do you collect different samples?

Answer explained in pre analytical phase.

### **3.6 Catalase test**

This is the first test done after the isolate is given on nutrient agar slope. This test is used to differentiate those bacteria that produce the enzyme catalase, such as *Staphylococci*, from non-catalase producing bacteria such as *Streptococci*.

**Principle:** Catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. Catalase is an enzyme produced by bacteria. The enzyme splits hydrogen peroxide into water and nascent oxygen. Hydrogen peroxide is a byproduct of aerobic respiration and is lethal if it accumulates in the bacterial cell. Catalase degrades the hydrogen peroxide present in the bacterial cell before it can do any damage to the cell. Bubbles of oxygen are released if the organism is a catalase producer. The culture should not be more than 24 hours old. The lack of catalase is evident by a lack of or weak bubble production. The catalase test is most often used to differentiate *Staphylococcus spp.* from *Streptococcus spp.* and also to differentiate *Bacillus spp.* (positive) from *Clostridium tertium* (negative). There are three types of test. Slide test and tube test and plate test. Ideally slide test is not recommended as it is harmful to us due to nascent organisms released during the reaction.

#### **Slide test**

Place a drop of 3% hydrogen peroxide onto the slide

Use a loop or sterile wooden stick to transfer a small amount of colony growth to the surface of slide containing 3% hydrogen peroxide.

Observe for the evolution of oxygen bubbles immediately.

#### **Tube test**

Pour 2–3 ml of the 3% hydrogen peroxide solution into a test tube.

Using a sterile wooden stick or a glass rod (not a nichrome wire loop), remove several colonies of the test organism and immerse in the hydrogen peroxide solution.

**Important:** Care must be taken when testing an organism cultured on a medium containing blood because catalase is present in red cells. If any of the blood agar is removed with the organism, a false positive reaction may occur.

Look for immediate bubbling immediately.

### **Plate catalase test**

Here on the culture plate place a drop of 3% hydrogen peroxide on the colonies and close the lid immediately. Look for brisk effervescence immediately.

### **Quality control:**

Positive control: *Staphylococcus aureus* ATCC 25923

Negative: ATCC *Streptococcus pyogenes* / *Enterococcus faecalis* ATCC 29212

### **Questions**

1. What is catalase test?

Catalase is an enzyme produced by bacteria. The enzyme splits hydrogen peroxide into water and oxygen. Hydrogen peroxide is a by-product of aerobic respiration and is lethal if it accumulates in the bacterial cell. Catalase degrades the hydrogen peroxide present in the bacterial cell before it can do any damage to the cell. The catalase test is most often used to differentiate *Staphylococcus spp.* from *Streptococcus spp.* and to differentiate *Bacillus spp* (positive) from *Clostridium tertium* (negative).

2. What are the different types of catalase test?

Slide catalase test, Tube catalase test, Plate catalase test.

3. What is the percentage of hydrogen peroxide used?

The percentage of hydrogen peroxide used is 3%.

4. Which test is better and why?

Tube catalase test is better as there is possibility of aerosol transmission of organisms in slide test.

5. How do you perform catalase test and what precautions should you take?

It can be done by both slide and tube catalase method, but tube catalase test is preferred over slide test as there are chances of aerosol transmissions in slide test. If slide test is performed it should be done inside the biosafety cabinet.

6. What is the positive control and negative control used in catalase test?

Positive control *S.aureus* ATCC 25923

Negative control *Streptococcus* spp. ~~*Enterococcus faecalis*~~ ATCC 29212.

7. Which loop is used for doing catalase test and why?

The test is done using sterile glass rod or sterile wooden stick and not nickel or chromium loop as it can cause false positive reaction.

8. How do you interpret the catalase test?

The test is interpreted by looking for brisk effervescence.

9. Give some examples of catalase positive and catalase negative organisms

<b>Catalase positive bacteria</b>	<b>Catalase negative bacteria</b>
<i>Staphylococcus</i> spp.	<i>Streptococcus pyogenes</i>
<i>Micrococci</i>	<i>Shigella dysenteriae</i> type I
<i>Corynebacterium diphtheriae</i>	<i>Gardnerella vaginalis</i>
<i>Enterobacteriaceae</i>	<i>Fusobacterium</i> spp
	<i>Eikenella corrodens</i>
	<i>Kingella kingae</i>

10. What is the percentage of hydrogen peroxide used in *Mycobacterium tuberculosis* and how do you interpret?

We use 30% hydrogen peroxide to differentiate *Mycobacterium tuberculosis* from *Atypical mycobacteria*.

The test is performed by mixing equal volumes of 30% hydrogen peroxide and 0.2% catechol in distilled water and then adding it to 5ml of a mycobacterial test culture. It is allowed to stand for a few minutes. The effervescence indicates catalase production. *Mycobacterium tuberculosis* is catalase negative and *atypical mycobacteria* is catalase positive.

11. Name some bacteria which are catalase negative but are members of Enterobacterales?

*Shigella dysenteriae* type 1 is catalase negative and belong to Enterobacterales.

12. Can you do catalase test from blood agar plate? If no, give reasons.

Catalase test should not be done from blood agar plate due to peroxidase enzyme present in erythrocytes.

13. What is the importance of doing catalase test in Gram negative bacilli?

This test helps in differentiating gram-negative bacilli which are catalase producers from bacilli which are not catalase producers.

14. If at all you need to do catalase from blood agar plate, how will you do?

In blood agar plate, false positive results can occur because of peroxidase present in erythrocytes. If at all it has to be done, make sure only the colonies alone are picked up.

15. What is heat stable catalase test in *Mycobacterium tuberculosis*?

This test is done to find out the ability of the catalase enzyme to remain active after heating in *Mycobacterium tuberculosis*. When heated at 68 deg for 20 minutes, catalase of *Mycobacterium tuberculosis*, *M. bovis* are inactivated. *Mycobacterium tuberculosis* is negative control and *Mycobacterium fortuitum* is positive control.

16. How do you do semiquantitative test?

Semiquantitative test is based on the relative activity of the enzyme which is determined by the height of the column of oxygen produced by action of untreated enzymes produced by the organism.

On the basis of semiquantitative catalase test, *Mycobacteria* are classified into 2 groups: Those that produce column less than 45mm height of bubbles and those that produce column more than 45mm height of bubbles. *Mycobacterium tuberculosis* produce a column of less than 45mm whereas *Mycobacterium kansasii* produce a column of more than 45mm.

17. What is the percentage of hydrogen peroxide used for anaerobic organisms?

The percentage of hydrogen peroxide used for anaerobic organism is 15%.

18. How does the reaction take place in anaerobic organisms?

The reaction takes place with the help of enzyme peroxidases present in organisms.

### 3.7. Oxidase test

The cytochromes are iron-containing hemoproteins that act as the last link in the chains of aerobic respiration by transferring electrons (hydrogen) to oxygen, with the formation of water. The cytochrome oxidase test uses the dye p-phenylene diamine dihydrochloride which acts as a substitute for oxygen as artificial electron acceptors. This enzyme oxidizes the reagent N-N tetramethyl para-phenylene diamine dihydrochloride (a colorless reagent in reduced form) to indophenol blue, a purplish-blue colored product.

There are two types of method. Wet method and dry method.

#### Dry filter paper method

1. Select a commercially available paper disk that has been impregnated with 1% tetramethyl-p phenylenediamine dihydrochloride.
2. Use a platinum wire or wooden stick to remove a small portion of a bacterial colony (preferably not more than 24 hours old) from the agar surface and rub the sample on the commercial disc.

Observe inoculated area of disk for a colour change to deep blue or purple within 10 seconds (timing is critical)

#### Wet filter paper method

Occasionally the test is performed by flooding the culture plate with oxidase reagent, but this technique is not recommended for routine use because the reagent rapidly kills bacteria. It can however be useful when attempting to isolate *N. gonorrhoeae* colonies from mixed cultures in the absence of a selective medium. The oxidase positive colonies must be removed and subcultured within 30 seconds of flooding the plate.

**Important:** Acidity inhibits oxidase enzyme activity; therefore, the oxidase test must not be performed on colonies that produce fermentation on carbohydrate containing media such as TCBS or MacConkey agar.

Sub inoculation on nutrient agar is required before the oxidase test can be performed. Colonies tested from a medium that contains nitrate may give unreliable oxidase test results.

### **Quality control**

Positive control: *Pseudomonas aeruginosa* ATCC 27853

Negative control: *Escherichia coli*. ATCC 25922

### **Questions**

1. What is oxidase test?

Oxidase test is done to detect the presence of an enzyme oxidase produced by the bacteria.

2. What is the name of oxidase reagent?

The oxidase reagent is N-N tetramethyl para-phenylene diamine dihydrochloride.

3. What is the principle of oxidase test?

The cytochromes are iron containing haemoprotein that act as the last link in the chains of aerobic respiration by transferring electrons (hydrogen) to oxygen, with the formation of water. The cytochrome oxidase test uses the dye p-phenylene diamine dihydrochloride which acts as a substitute for oxygen as artificial electron acceptors. This enzyme oxidises the reagent N-N tetramethyl para-phenylene diamine dihydrochloride (a colourless reagent in reduced form) to indophenol blue, a purplish-blue coloured product.

4. When do you interpret the test?

The test should be interpreted within 10 sec. Any reaction developing after 10 sec is considered negative.

5. What is the positive control and negative control of oxidase test?

Positive control: *Pseudomonas aeruginosa* ATCC 27853

Negative control: *Escherichia coli* ATCC 25922

6. What are the different methods of oxidase test?

There are two types of method. Wet method and dry method. Procedure explained above.

7. Why should you not use nickel or iron loop for interpreting the test?

The test should not be done with iron or nickel loop as small traces of iron will catalyse the reaction and give false positive results.

8. Why should you not do the test from Mac conkey agar?

When colonies are taken from Mac conkey agar, the pink, violet colour is due to carry over from the medium and it is not a true oxidase reaction. The true reaction gives an intense purple hue. Hence, it should not be done.

9. What is modified oxidase test?

The modified oxidase test is a rapid method to differentiate *Staphylococcus* from *Micrococcus spp.* by detection of the enzyme oxidase. Filter paper disks impregnated with tetramethyl-p-phenylenediamine in dimethyl sulfoxide 6%(DMSO) is used in order to detect the oxidase enzyme. DMSO serves for the permeability of cells to the reagent along with providing solubility and stability against auto-oxidation.

When exposed to atmospheric oxygen, the oxidase enzyme reacts with the oxidase reagent and cytochrome C to yield the coloured compound, indophenol indicated as blue or purplish-blue coloration on the disc after the bacterial colony is introduced on the disc.

10. What is the use of modified oxidase test?

This test is used to differentiate *Staphylococcus spp* from *Micrococcus spp.*

11. Give some examples of oxidase positive and oxidase negative organisms.

Oxidase positive	Oxidase negative
<b>Gram negative bacilli</b>	1. All genera in the family <i>Enterobacteriaceae</i>
1. <i>Pseudomonas spp.</i>	2. <i>Acinetobacter calcoaceticus</i>
2. <i>Vibrio spp.</i>	3. <i>Bordetella parapertusis</i>
3. <i>Aeromonas spp.</i>	4. <i>Gardenella vaginalis</i>
4. <i>Pleisomonas spp.</i>	5. <i>Franscieilla tularensis</i>
5. <i>Campylobacter spp.</i>	
6. <i>Bordetella spp. except Bordetella parapertusis</i>	
7. <i>Brucella spp. except Brucella canis.</i>	
<b>Gram negative cocci</b>	
1. <i>Neisseria spp.</i>	
2. <i>Branhamella spp.</i>	

### 3.8. Wet mount

Generally required to:

Examine specimens and cultures for motile bacteria.

Examine C.S.F. for capsulated yeast cells.

Examine specimens for fungi

In case of urine sample, it gives information about the pus cells, casts, RBCs and epithelial cells. Take a loop full of urine with an inoculum loop. The inoculum loop comes with 4mm diameter or 2mm diameter. Take a loop-full on a clean slide, keep a cover slip and examine under 40X objective.

You need to use cavity slide for examination.

### **3.9. Hanging drop preparation**

1. Place a small drop of suspension on a slide and cover with a cover glass. Avoid making the preparation too thick. It is advisable to seal the preparation with nail varnish or molten petroleum jelly to prevent it drying out. Placing a drop of suspension on a cover glass and inverting this over a cavity slide or over a normal slide supported on a ring of plasticine is not recommended. Vibration of the fluid makes the preparation difficult to examine.

2. Examine the preparation microscopically for motile organisms, using the 10X and 40X objectives. Make sure the iris diaphragm of the condenser is sufficiently closed to give good contrast otherwise the organisms will not be seen. First see the drop under 4X. You need to locate the drop. Then using 10X bring the edge of the drop in middle. Then without adjusting change to 40X. This will bring the edge of the drop in correct position. Then do the final adjustment alone.

**Note:** The movement of small motile bacteria must be distinguished from the on-the-spot vibratory movement (Brownian movement) which is random movement of small bodies suspended in fluid due to irregularities in their bombardment by molecules of water. True bacterial motility is the displacement of position i.e the organism moves from one place to another in different directions and change their positions in the field.

### **Questions**

1. When do you do hanging drop?

Hanging drop is done mainly to check the motility of the bacteria whether, motile or non-motile.

2. How do you do hanging drop?

The answer is explained in the procedure above.

3. What are all the adjustments done in microscope to do hanging drop?

You need to use only 40X objective for hanging drop. Lower the condenser, partially close the diaphragm.

4. Give some examples of motile and non-motile bacteria.

Motile—*Escherichia coli*, *Salmonella* spp, *Pseudomonas aeruginosa*, *Enterobacter* spp,

Non motile—*Klebsiella* spp, *Shigella* spp, *Salmonella gallinarum*, *Salmonella pullorum*.

5. Give some examples of motile gram-positive organisms.

*Vegococcus*, *Rhodococcus*, *Enterococcus caesaliflavus*,

6. What are the different types of motility?

Darting motility—*Vibrio cholerae*, *Campylobacter*

Tumbling motility—*Listeria monocytogenes*

Falling leaf motility—*Giardia lamblia*

Corkscrew motility—*Spirochaetes*.

Stately motility—*Clostridia*, *Bacillus* spp.

Swarming motility—*Clostridium tetani*, *Proteus* spp.

Gliding motility—*Mycoplasma*

Corkscrew, lashing, flexion extension motility—*Spirochaetes*

Football motility—*Balantidium coli*

*Yersinia enterocolitica* is motile at 22 deg C and non-motile at 37 deg C.

*Listeria monocytogenes* is motile at 25 deg C and non-motile at 37 deg C

7. Why do you focus the edge of the drop?

Edge of the drop is focused to observe for motility and morphology for the following reasons. Concentration of organisms is more at edge, because:

Aerobic organisms tend to move towards edge

Surface tension is less at edge and hence concentration of organisms is more

Density is less at the edge of the drop, hence contrast is better and organisms are clearly visible.

8. How do you differentiate brownian movement from motility?

Brownian movements are random oscillatory movements due to convection currents. True bacterial motility is the ability of an organism to move itself in different directions or a single direction.

9. Where else do you use 40X objective?

To view stool examination both saline and iodine mount, wet mount of urine specimen, negative stain to view the capsulated organism.

10. How do you demonstrate bacterial motility?

Wet mount—Hanging drop experiment.

Microscopic methods—Dark ground microscopy, Phase-contrast microscopy

Macroscopic methods—Noting swarming growth on agar medium,

Use of semi solid agar-mannitol motility medium (0.2%), Craige's tube- or U-shaped tube.

Demonstration of flagellae-- Silver impregnation methods

Special stains such as Leifson's stain

Electron microscope

### 3.10 Indole test

**Principle:** Tryptophan is an amino acid. This is present in peptone water or tryptophane broth of the

culture medium and when acted upon by the enzyme tryptophanase, it is converted into indole, pyruvic acid and ammonia.

The indole reacts with aldehydes to produce a red coloured product. The reagents used are

**Kovac's reagent:** consists of para-dimethyl amino-benzaldehyde, isoamyl alcohol, concentrated hydrochloride.

**Ehrlich's reagent:** consists of para-dimethyl amino benzaldehyde, absolute ethyl alcohol and concentrated HCl.

In a positive test, a red-violet ring develops within minutes of addition of Kovac's reagent. Negative reaction is indicated by the developing a yellow ring.

**Note:** Ehrlich's reagent is more sensitive for detecting small amounts of indole. The procedure when using Kovac's reagent and Ehrlich's reagents are as follows.

## **Method**

### **For Enterobacterales**

1. Inoculate the organisms in peptone water / tryptophan broth and incubate at 35°-37°C in ambient air for 24 hours. Add 0.5 mL of Kovac's reagent to the broth culture.

### Other Gram-Negative Bacilli

Inoculate the organisms in peptone water and incubate at 35°-37°C in ambient air for 24 hours.

2. Add 1 mL of xylene to the culture.

Shake the mixture vigorously to extract the indole and allow it to stand until the xylene forms a layer on top of the aqueous phase.

Add 0.5 mL of Ehrlich's reagent down the side of the tube.

## **Results:**

Positive- Pink to wine colored ring after addition of appropriate reagent

Negative- No color change after addition of the appropriate reagent.

### **Spot indole test.**

Small portion of the colony is transferred from a non-selective medium such as blood or chocolate agar to a strip of filter paper that has been saturated with Kovac's reagent or p-dimethyl amino benzaldehyde solution.

The immediate development of a red colour indicates the presence of indole and a positive test.

## **Quality Control**

Positive control: *Escherichia coli* ATCC 25922

Negative control: *Klebsiella pneumoniae*

## **Questions**

1. What is the use of indole test?

Indole test is done to detect the presence of tryptophanase present in bacteria.

2. What is the positive control and negative control used?

Positive control: *Escherichiae coli* ATCC 25922

Negative control: *Klebsiella pneumoniae*

3. What are the end products of tryptophan metabolism?

The end products are indole, skatole and indole acetic acid.

4. What are the reagents used in this test?

The reagents used are Kovac's reagent and Ehrlich's reagent.

5. What are the constituents of Kovac's reagent?

Kovac's reagent contains P-dimethyl amino benzaldehyde, isoamyl alcohol and conc. Hydrochloric acid.

6. What are the constituents of Ehrlich's reagent?

Ehrlich's reagent contains P-dimethyl amino benzaldehyde, absolute ethyl alcohol and conc. Hydrochloric acid.

7. How to test indole production by adding Ehrlich's reagent?

Take 0.5ml of culture broth and mix with equal volume of Ehrlich's reagent. Shake and allow it to stand for few minutes and then observe the result.

Positive reaction is indicated by development of red-violet ring and negative by yellow ring.

This can also be tested in another method by adding 0.5-1ml of xylene or ether to the culture broth and

shaking it well. Then 0.5ml of Ehrlich's reagent is added and the result read immediately.

8. Why does the ring float in Indole test?

It is because of the reaction with aldehyde.

9. How do you do spot indole test?

Small portion of the colony is transferred from a non-selective medium such as blood or chocolate agar to a strip of filter paper that has been saturated with Kovac's reagent or p-dimethyl amino benzaldehyde solution. The immediate development of a red colour indicates the presence of indole and a positive test.

10. Which reagent is better when there is small production of indole?

Ehrlich's reagent is more sensitive when there is small production of indole and in non-fermenters.

11. Give some examples of indole positive and indole negative bacteria.

<b>Indole positive</b>	<b>Indole negative</b>
<i>Escherichia coli</i>	<i>Escherichia vulnaris</i>
<i>Klebsiella oxytoca</i>	<i>Klebsiella pneumoniae</i>
<i>Proteus vulgaris</i>	<i>Proteus mirabilis</i>
<i>Providentia rettgeri</i>	<i>Salmonella typhi</i>
<i>Morganella morganii</i>	<i>Salmonella paratyphi A</i>
<i>Vibrio cholerae</i>	<i>Salmonella paratyphi B</i>
<i>Aeromonas hydrophilia</i>	<i>Shigella sonnei</i>
<i>Citrobacter koseri</i>	

### 3.11. Methyl red and Voges Proskauer test

#### Methyl red test

**Purpose:** The combination test methyl red (MR) and Voges-Proskauer (VP) differentiates members of the Enterobacteriaceae family.

**Principle:** The methyl red test is a quantitative test for acid production, requiring positive organisms to produce strong acids (lactic acid, acetic acid, formic acid) from glucose through **the mixed acid pathway**. Many species of Enterobacteriaceae produce sufficient quantities of strong acids that can be detected by Methyl red indicator. Only the organisms that can maintain this low pH after prolonged incubation (48-72 hours) can give positive reaction. The MR indicator is added after incubation. Methyl red is red at pH 4.4 and yellow at pH 6.2. A clear red is a positive result. Yellow is a negative result.

**Media:** Glucose phosphate broth: Peptic digest of animal tissue (3.5 g), pancreatic digest of casein (3.5g), dextrose (5 g), K<sub>2</sub>HPO<sub>4</sub> (5 g), per 1000 mL, pH 6.9.

#### Method

Inoculate MRVP broth with 1 drop from a 24-hour brain-heart infusion broth culture.

Incubate at 35°-37°C for a minimum of 48 hours in ambient air.

Tests should not be made with cultures incubated less than 48 hours, because the end products build up to detectable levels over time.

### **Methyl red (MR ) test**

1. Add 5 or 6 drops of methyl red reagent per 5 mL of broth.

2. Read reaction immediately.

Expected Results

Positive: Bright red color, indicative of mixed acid fermentation

Negative: Yellow color

Quality control

Positive control: *E.coli* ATCC 25922

Negative control: *Klebsiella aerogenes*.ATCC 13048

### **3.12. Voges proskaeur test**

Bacteria that utilises butaendiol pathway have an enzyme that condenses two pyruvate molecules to CO<sub>2</sub> and alpha aceto lactate. This is then decarboxylated resulting in acetoin formation. In the presence of hydrogen ions, acetoin is further reduced to 2,3, butanediol. The VP detects the organism's ability to convert the acid products to acetoin and 2,3-butanediol. Organisms capable of using the VP pathway produce a smaller amount of acid during glucose fermentation and therefore do not produce a color change when the methyl red indicator is added.

**Media:** Glucose phosphate broth: Peptic digest of animal tissue (3.5 g), pancreatic digest of casein (3.5 g), dextrose (5 g), K<sub>2</sub>HPO<sub>4</sub> (5 g), per 1000 mL, pH 6.9.

**Method 1.** After incubation at 37 deg for 48 hours, add 0.5 ml of O'Meara reagent (40g potassium hydroxide and 0.3g creatine in 100ml distilled water). Place tubes in water bath for 4 hours. Aerate by shaking at intervals. A positive reaction is denoted by the development of eosin-pink colour, usually in 2-5min.

**Method 2.** After incubation at 37 deg C for 48 hours, add 1ml of 40% potassium hydroxide and 3ml of 5% solution of  $\alpha$ - naphthol in absolute ethanol. A positive reaction is indicated by the development of pink colour in 2-5 min, becoming crimson in 30 min. The tube can be shaken at intervals to ensure maximum aeration.

**Quality control**

Positive control: *Klebsiella aerogenes*.ATCC 13048

Negative control: *E.coli* ATCC 25922

## Questions

1. What is the significance of MR, VP test?

MR test detects the product of stable high concentration of acidic end products.

VP test shows the presence of non-acidic neutral end product acetyl methyl carbinol.

2. What is the principle of MR test?

The methyl red test is a quantitative test for acid production, requiring positive organisms to

produce strong acids (lactic acid, acetic acid, formic acid) from glucose through the mixed acid pathway. Many species of Enterobacteriaceae produce enough strong acids that can be detected by Methyl red indicator. Only the organisms that can maintain this low pH after prolonged incubation (48-72 hours) can give positive reaction.

3. What is the indicator used in MR test and what is the pH range?

The indicator used is Methyl red and the pH with a range between yellow 6.0 and red 4.4.

4. What are the positive and negative controls used in MR test?

Positive control: *E.coli* ATCC 25922

Negative control: *Klebsiella aerogenes*.ATCC 13048.

5. When do you do MR test?

The test is done after incubation of the test broth for 48 hours because the organisms have to produce sufficient quantities of acid and it has to be maintained in the same acidic pH which turns the methylred to red colour.

6. Give some examples of MR positive and MR negative organisms.

<b>MR positive bacteria</b>	<b>MR negative bacteria</b>
<i>Escherichia coli</i>	<i>Klebsiella pneumoniae</i>
<i>Klebsiella ozaenae</i>	<i>Enterobacter spp.</i>
<i>Edwardsiella spp.</i>	
<i>Citrobacter spp.</i>	
<i>Proteae spp.</i>	
<i>Salmonella spp.</i>	

7. Can you give some examples of VP positive and VP negative organisms.

<b>VP positive bacteria</b>	<b>VP negative bacteria</b>
<i>Klebsiella pneumoniae</i>	<i>Escherichia coli</i>
<i>Enterobacter cloacae</i>	<i>Edwardsiella tarda</i>
<i>Serratia marcescens</i>	<i>Salmonellae</i>
<i>Vibrios cholerae ( Eltor)</i>	<i>Proteae spp.</i>
	<i>Yersinia spp.</i>

8. What is the positive control and negative control used in VP test?

Positive control *Klebsiella aerogenes*. ATCC 13048

Negative control *E.coli* ATCC 25922

9. Where do you get both MR and VP positive and why so?

*Eltor Vibrio*, *Yersinia enterocolitica*, *Staphylococcus aureus*, because they use both the pathways (mixed acid and butanediol pathway).

10. Mention different metabolic pathways with examples.

Homolactic fermentation eg. *Streptococcus*, *Enterococcus*, *Pediococcus* and *Lactobacilli*.

Heterolactic fermentation—*Leuconostoc spp.*, yeasts

Mixed acid fermentation—*E.coli*, *Proteae*, *Citrobacter spp*

Butanediol fermentation—*Enterobacter* group of Enterobacteriaceae

Butanol fermentation—*Clostridium*

Propionic acid fermentation—*Bacterioides* and *propionobacterium*.

11. How much time does it take to interpret VP results and what precautions to be taken?

Answer explained in method 1 and 2.

### **3.13 Mannitol motility test**

**Principle:** These tests are used to determine if an organism ferments mannitol and is motile.

An organism must possess flagella to be motile.

#### **Method: Stab Culture Method**

Positive: Motile organisms will spread out into the medium from the site of inoculation.

The medium turns yellow when mannitol is fermented.

Negative: Non-motile organisms remain at the site of inoculation

#### **Procedure:**

Touch a straight needle to a colony of a young (18 to 24 hour) culture growing on agar medium. Stab once to a depth of only 1/3 to 1/2 inch in the middle of the tube.

Incubate at 35 deg C to 37 deg C.

#### **Quality Control:**

Positive: *Escherichia coli* ATCC 25922

Negative: *Klebsiella pneumoniae* ATCC 700603

### **Questions**

1. What is the percentage of agar used in mannitol motility test?

The percentage of agar used is 1.5%.

2. What is the indicator used and pH range?

The indicator used is phenol red and the pH ranges from 6.5 to 8.2.

3. What are all the other tests that can be done to detect the motility of the organisms?

Hanging drop, Mannitol motility medium, Craige's tube test to test the motility.

4. Mention some medias that use phenol red as indicator.

TSI test, Mannitol motility test, Urease test, XLD (xylose lysine deoxycholate agar), viral transport medium, tissue culture flask for virus cultivation.

5. What are the precautions to be taken while inoculating in mannitol motility medium?

While stabbing in the medium, make sure you don't touch the bottom of the tube since it can create an anaerobic environment.

### 3.14. Triple sugar iron test (TSI)

It is used to determine whether a gram-negative bacillus utilizes glucose, lactose or sucrose fermentatively and forms hydrogen sulfide ( $H_2S$ ). TSI contains 10 parts lactose: 10 parts sucrose: 1 part glucose and peptone. Phenol red and ferrous sulfate serve as indicators of acidification and  $H_2S$  formation.

**Principle:** The TSI agar is distributed in the tube which contains a slant and a butt. TSI medium indicates whether the bacteria ferments glucose only, or lactose and sucrose also with or without production of gas. The medium can detect production of hydrogen sulphide ( $H_2S$ ) as well as the bacteria which utilizes only glucose (but not lactose or sucrose). Due to acid production, the colour of the phenol red (indicator) is changed to yellow and the whole medium appears yellow in colour. After further incubation as the glucose is fully exhausted, the bacteria begins to oxidatively degrade the amino acid present in the medium. Since oxygen is exposed only to the slant portion, the oxidative degradation results in change of colour to red. In the butt portion, the amino acid degradation is insufficient to overcome the acid formed, so medium remains yellow in colour. If the TSI medium is inoculated with lactose fermenting organism, then even after the glucose is completely used up in first 8-12 hours, fermentation continues as the organism is able to utilise lactose which is 10 times that of glucose. So the slant and butt appears yellow. For the detection of  $H_2S$  which is a colourless gas, medium must include an indicator to detect  $H_2S$ . Sodium thiosulfate is the source of sulfur atoms. Ferrous sulfate is the indicator used for the detection of  $H_2S$  which is indicated by the production of black precipitate.

#### Procedure

With a straight inoculation needle, touch the top of a well-isolated colony.

Inoculate TSI by first stabbing through the centre of the medium to the bottom of the tube and then streaking the surface of the agar slant.

Leave the cap on loosely and incubate the tube at 35 deg C in ambient air for 18 to 24 hours

**Note:** A black precipitate in the butt indicates production of ferrous sulphide and H<sub>2</sub>S gas (H<sub>2</sub>S+).

Bubbles or cracks in the tube indicate the production of gas.

### **Quality Control**

A/A : *Escherichia coli* ATCC 25922

K/A H<sub>2</sub>S+: *S Typhi*

K/K: *Pseudomonas aeruginosa* ATCC 27853

K/A H<sub>2</sub>S -ve *Shigella spp.*

### **Questions**

1. What is TSI test?

Triple sugar iron test is to test the ability of the organism to utilize glucose, lactose and sucrose and also to check the ability to produce hydrogen sulphide.

2. What is the composition of TSI?

TSI contains beef extract, yeast extract, peptone, lactose 10g, sucrose 10g and glucose 1gm, ferrous sulphate, sodium chloride, sodium thiosulfate, Phenol red.

3. What is the source of sulphur?

The source of sulphur is sodium thiosulfate.

4. Give some examples of H<sub>2</sub>S producing organisms

*S. Typhi*

*S. Paratyphi B*

*Proteus vulgaris*

*Proteus mirabilis*

*Edwardsiella tarda*

*Citrobacter freundii*

*Shewanella putrefaciens*

*Campylobacter sputorum*

*Erysiphilothrix rhusiopathiae*

5. When do you interpret the reading of TSI test and why?

The readings of TSI test are taken only after 18-24 hours. The reason behind this is that the glucose concentration is less than that of lactose and sucrose. Glucose fermenters break down glucose first, then lactose. If the organism is non lactose or non-sucrose fermenter, the organisms metabolise peptones aerobically for their energy, producing alkaline end products. In the first 6-8 hours the whole tube will be acidic and will be yellow in colour. Production of alkaline end products results in the change of pH alkaline side and pink colour develops in the slant. So the pH of the slant is alkaline after 8 hours and the results are read only after 18 to 24 hours.

6. What is the principle behind this test?

The TSI agar is distributed in the tube which contains a slant and a butt. TSI medium indicates whether the bacteria ferments glucose only, or lactose and sucrose also with or without production of gas. The medium can detect production of hydrogen sulphide (H<sub>2</sub>S) as well as the bacteria which utilizes only glucose (but not lactose or sucrose). Due to acid production, the colour of the phenol red(indicator) is changed to yellow and the whole medium appears yellow in colour. After further incubation as the glucose is fully exhausted, the bacteria begins to oxidatively degrade the amino acid present in the medium. Since oxygen is exposed only to the slant portion, the oxidative degradation results in change of colour to red. In the butt portion, the amino acid degradation is insufficient to overcome the acid formed, so medium remains yellow in colour. If the TSI medium is inoculated with lactose fermenting organism, then even after the glucose is completely used up in first 8-12 hours, fermentation continues as the organism is able to utilise lactose which is 10 times that of glucose. So the slant and butt appears yellow. For the detection of H<sub>2</sub>S which is a colourless gas, medium must include an indicator to detect H<sub>2</sub>S. Sodium thiosulfate is the source of sulfur atoms. Ferrous sulfate is the indicator used for the detection of H<sub>2</sub>S which is indicated by the production of black precipitate.

7. Mention other indicators of H<sub>2</sub>S production.

Ferrous sulfate, Ferric ammonium citrate, Lead acetate, peptonized iron.

8. Mention the list of media used for detecting production of hydrogen sulfide.

Bismuth sulfite agar, Deoxy cholate citrate agar, Lysine iron agar, TSI agar, Kligler iron agar, Lead acetate agar, Salmonella-shigella agar, Xylose lysine deoxycholate agar, Wilson blair bismuth sulfite agar.

9. What is length of butt and slant used in TSI?

We use 3cm butt and 3 cm slant in TSI.

10. Give some examples of bacteria with different combinations of interpretations in TSI.

*E.coli* A/A gas + and H<sub>2</sub>S -ve

*S.Typhi* K/A gas -ve, H<sub>2</sub>S specks

*Shigella* K/A Gas + H<sub>2</sub>S -ve

*Proteus mirabilis* K/A gas +H<sub>2</sub>S abundant

*Pseudomonas aeruginosa* K/K gas +, H<sub>2</sub>S -ve

11. How does Kligler iron agar differ from TSI?

Kligler iron agar differs from TSI in that it does not contain sucrose. It contains only glucose and lactose whereas TSI contains glucose, lactose and sucrose. In case of *Yersinia* we prefer KIA as *Yersinia* does not ferment sucrose and the reaction will be K/A in KIA whereas it will be A/A in TSI

12. Name different methods used for demonstration of H<sub>2</sub>S production?

TSI test, Kligler iron agar test, lead acetate strips test, Salmonella shigella agar medium, XLD, DCA agar, WBBS medium, Lysine iron agar, Citrate sulphide agar, Sulphur indole motility medium.

13. What other special sugars do you know which are used in diagnostic microbiology?

Glucose, lactose, maltose, mannitol, sucrose, xylose, arabinose, raffinose.

14. How do you interpret the result in non-fermenters?

In non-fermenters there is no utilization of carbohydrates and so only the amine production in the slant alone makes the medium alkaline which produces a red color throughout the medium.

15. How do you detect H<sub>2</sub>S production with lead acetate strip?

Lead acetate is a sensitive indicator and should be used when bacteria produces trace amounts of H<sub>2</sub>S. This can be done by draping a lead acetate impregnated strip under the cap of KIA medium because when lead acetate is incorporated in the media it inhibits the growth of most fastidious bacteria.

### 3.15. Urease test

This test is used to determine the ability of an organism to produce the enzyme urease, which hydrolyzes urea. Hydrolysis of urea produces ammonia and CO<sub>2</sub>. The formation of ammonia alkalizes the medium, and the pH shift is detected by the color change of phenol red from light orange at pH 6.8 to magenta at pH 8.1.

There are two types of media: Christensen's urease agar and Stuart's urea broth.

**Method:** Christensen's urease agar method

Positive: Change in color of slant from light yellow to magenta pink

Negative: No color change (agar slant and butt remain light yellow)

**Specimen:** 18–24-hour culture growth

Streak the surface of a urea agar slant with a portion of a well-isolated colony or inoculate slant with 1 to 2 drops from an inoculated culture broth.

Leave the cap on loosely and incubate tube at 35 deg C in ambient air for 48 hours to 7 days.

### **Quality Control:**

Positive: *Proteus vulgaris*

Negative: *Escherichia coli* ATCC 25922

1. What is urease test?

Urease test is done to detect the presence of urease enzyme produced by bacteria and fungi.

2. What is the principle of urease test?

Urease is a diamide of carbonic acid. Urease which is the enzyme produced by the bacteria and fungi hydrolyses urea and releases ammonia and carbon dioxide. Ammonia reacts in solution to form ammonium carbonate, which results in increase in pH of the medium and the phenol red indicator changes to magenta pink.

3. What is the positive control and negative control used in urease test?

Positive control: *Proteus vulgaris*

Negative control: *Escherichia coli* ATCC 25922

4. What are the different types of media for the detection of urease test?

We have two media for the detection of urease test: Christensen's agar which is an agar-based media, Stuart's broth which is a liquid-based media.

5. How do you interpret the test in these two?

Christensen's urea agar is a solid medium whereas Stuart's broth is a liquid medium used for testing the urease activity of the bacteria.

In Christensen's urea agar, purple, pink colour is seen on the slant which indicates alkalization and urea hydrolysis.

In Stuart's broth, purple, pink colour is seen throughout the medium which indicates alkalization and urea hydrolysis. This can be assessed by the turbidity in the medium.

In general we use agar based media as it is less buffered and permits the detection of smaller amounts of ammonia. This media can be used for organisms that produce less urease like *Klebsiella*, *Enterobacter*, *Brucella* and *Bordetella bronchiseptica*. In broth media, it is heavily buffered with phosphate salts at a pH of 6.8. Relatively large quantities of ammonia must be formed by the organism before the buffer system is overcome and the pH is elevated above 8.0 to produce a color change. Hence, it is virtually selected for *Proteus spp.*

6. Name the slow, rapid and most rapid urease producing bacteria.

Strong or most rapid urease producers *Brucella spp.* *Helicobacter pylori*

**Rapid urease producers** *Proteus spp.*, *Morganella spp.*

**Slow urease producers** *Klebsiella spp.*, *Enterobacter spp.*

**Urease producing fungi** *Cryptococcus neoformans*, *Trichophyton mentagrophytes*

7. List an important test of urease test in morphology.

Urease test in mycology is mainly used for the identification *Cryptococcus spp.*

8. Give some examples of urease negative organisms.

*E. coli*, *Salmonella spp.*, *Shigella spp.*

### 3.16. Citrate test

#### Principle

This test is used to determine the ability of an organism to utilize sodium citrate as its only carbon source and inorganic ammonium salts as its only nitrogen source. Bacteria that can grow on this medium turn the bromothymol blue indicator from green to blue.

In the absence of fermentable glucose or lactose, some bacteria are capable of using citrate as a sole source of carbon for their energy. Citrate is acted by the

enzyme citrate permease which converts citrate to pyruvate to carbon dioxide. During this reaction the medium becomes alkaline, the CO<sub>2</sub> generated combines with sodium and water to form sodium carbonate, an alkaline product.

The bromothymol blue changes colour from green to blue (pH from 6.9 to 7.6).  
Simmon's citrate and Koser's citrate are two examples of citrate used.

Positive: Development of deep blue colour within 24-48 hours of inoculation.

Negative: Absence of growth, no change in colour.

Specimen: 18-24-hour culture growth.

### **Procedure:**

Inoculate Simmons citrate agar lightly on the slant by touching the tip of a inoculation wire to a colony that is 18 to 24 hours old. You need to have light inoculum as heavy inoculum can cause false positive results.

Incubate at 35 deg C to 37 deg C

Observe for development of blue color, denoting alkalization.

### **Quality control**

Positive control: *Klebsiella pneumoniae* ATCC 700603

Negative control: *Escherichia coli* 25922

### **Questions**

1. What is citrate test?

Citrate test is the test to detect an enzyme produced by bacteria.

2. What is the principle of citrate test?

In the absence of fermentable glucose or lactose, some bacteria are capable of using citrate as a sole source of carbon for their energy. Citrate is acted by the enzyme citrate permease which converts citrate to pyruvate to carbon dioxide. These products are then enzymatically converted to pyruvic acid and carbon dioxide (CO<sub>2</sub>). During this reaction the medium becomes alkaline, the CO<sub>2</sub> generated combines with sodium and water to form sodium carbonate, an alkaline product. The bromothymol blue changes colour from green to blue (pH from 6.9 to 7.6).

3. Give some examples of citrate positive and citrate negative organisms.

<b>Citrate positive</b>	<b>Citrate negative</b>
<i>Klebsiella pneumoniae</i>	<i>Escherichia coli</i>
<i>Citrobacter spp.</i>	<i>Salmonella Typhi</i>
<i>Proteus mirabilis</i>	<i>S. ParaTyphi A</i>
<i>Klebsiella cloacae</i>	<i>Shigella spp</i>
<i>Serratia marcesens</i>	<i>Edwardsiella tarda</i>
<i>Pantoea</i>	

4. When do you inoculate for citrate test and what is the inoculum thickness?

You need to inoculate citrate first before inoculating any other media to prevent carry over of proteins or carbohydrates from other media and the inoculum should be very thin. If the inoculum is thick it can cause false positive reactions.

5. What is the indicator used and where else do you use the same indicator?

The indicator used is bromothymol blue. It is also used in CLED, TCBS agar and carbohydrate fermentation test.

6. What is the positive control and negative control used in citrate test?

Positive control: *Klebsiella aerogenes*

Negative control: *E. coli* ATCC 25922.

7. Mention different kinds of citrate utilization tests.

Simmon's citrate and Koser's citrate utilization test.

8. Difference between Simmon's citrate and Koser's citrate.

<b>Simmon's citrate</b>	<b>Koser's citrate</b>
It is a slant (solid) medium	It is a liquid (broth) medium
The medium contains bromothymol blue as indicator	It does not contain any indicator
Positive test is indicated by growth and change in colour of the indicator to blue	Positive test is indicated by presence of turbidity in the medium

### 3.17. Nitrate reduction test

#### Principle

Bacterial demonstration of nitrate reduction have the capability of extracting oxygen from nitrates to nitrites and other products. The presence of nitrites in the test medium is detected by the addition of alpha naphthylamine and sulphanilic acid, with the formation of red diazonium dye p-sulfobenzen-azo-alpha naphthylamine.

#### Procedure

In the overnight culture of organism in nitrate broth, add an equal volume of alpha naphthylamine and sulphanilic acid (0.5ml each). Observe for the development of brick red colour immediately within few minutes of adding the reagents.

#### Quality Control

Positive control: *E.coli* ATCC 25922

Negative control: *Acinetobacter baumannii*

If no colour develops after adding the test reagents, it is taken as negative test. It might have been reduced to products other than nitrites such as molecular nitrogen, nitric oxide or nitrous oxide. In this case, the reaction may show a false negative reading. Thus, it is necessary to add a small quantity of zinc dust to all negative reactions. Zinc dust reduces nitrates to nitrite and development of red colour after adding zinc dust indicates the presence of residual nitrates and confirms a true negative reaction.

#### Questions

1. What is the principle of nitrate reduction test?

The organisms produce nitrate reductase, which converts the nitrate (NO<sub>3</sub>) to nitrite (NO<sub>2</sub>). The reduction of nitrate to nitrite is determined by adding sulfanilic acid and alpha-naphthylamine. The sulfanilic acid and nitrite react to form a diazonium salt. The diazonium salt then couples with the alpha-naphthylamine to produce a red, water-soluble azo dye. If no color change occurs, the organism did not reduce nitrate or reduced it further to NH<sub>3</sub>, NO, or N<sub>2</sub>O<sub>2</sub>. Zinc is added at this point. If nitrate remains, the zinc will reduce the compound to nitrite and the reaction will turn positive, indicating a negative test result for nitrate reduction by the organism. If no color change occurs after the addition of zinc, this indicates that the organism reduced nitrate to one of the other nitrogen compounds previously described.

2. What are the ingredients present in nitrate broth?

It contains peptone, potassium nitrate and distilled water.

3. What is the positive control and negative control for nitrate reduction test?

Positive control: *E.coli* ATCC 25922

Negative control: *A.baumannii*.

4. What is the purpose of adding zinc dust to all negative reaction?

Negative reaction in nitrate reduction test may be of 2 types. One is true negative reaction in which nitrates are not reduced to nitrites. The other is false negative reaction, where the nitrates are reduced to nitrites and the development of red colour after adding zinc dust indicates a presence of residual nitrates and confirms a true negative reaction.

5. How will you avoid false negative reaction? Explain.

The false negative reaction can be avoided by adding zinc dust. If the result is still, it indicates false negative reaction.

6. What are the reagents added in the reactions?

Reagent A. Alpha naphthylamine

Reagent B. Sulphanilic acid

Add equal amounts to the culture broth and observe for brick red colour immediately.

7. How do you interpret nitrate reduction test?

The test is interpreted by adding an equal volume of alpha naphthylamine and sulphanilic acid (0.5ml each) to an overnight broth culture and observe for the development of brick red colour immediately within few minutes of adding the reagents.

8. Name some conditions when positive nitrate reduction test gives as negative reaction.

If the organism did not reduce nitrate or reduced it further to  $\text{NH}_3$ ,  $\text{NO}$ , or  $\text{N}_2\text{O}_2$  it can give negative reaction.

9. What will you do to find out that it is not negative but true positive test?

One is true negative reaction in which nitrates are not reduced to nitrites. The other is false negative reaction, where the nitrates are reduced to nitrites and the development of red colour after adding zinc dust. It indicates a presence of residual nitrates and confirms a true negative reaction.

### 3.18. Carbohydrates fermentation test

Bacteria differ widely in their ability to metabolize carbohydrates and related compounds.

The majority are tests done simply for the production of acid and gas or acid alone when a pure culture grows in the presence of the test compound. Peptone water with 1% sugar is added in the medium.

Commonly used sugars include the following.

Monosaccharides: Pentoses—arabinose, xylose, rhamnose

Hexoses-Glucose, fructose, mannose, sorbose, galactose

Disaccharides- Sucrose, maltose, lactose, trehalose, cellobiose

Trisaccharides—Raffinose

Polysaccharides- Starch, inulin, dextrin, glycogen

Polyhydric alcohols—Glycerol, adonitol, mannitol, dulcitol, sorbitol

Glycosides—Salicin, aesculin

#### Procedure

The test compound is usually added in the proportion of 0.5% to peptone water or 1.0% to serum bases. The indicators used are bromothymol blue (changes from blue to yellow), andrade's indicator (changes from yellow to pink). A small, inverted tube (durham tube), completely filled with liquid, containing no air bubbles is usually included in each culture tube or bottle to detect gas. Inoculate the organism from

peptone water into the sugars required for the test. The fermentation is indicated by the change in the colour of the medium and gas if produced will be present in the durhams tube.

### Questions

1. What is the percentage of sugar used in carbohydrate fermentation test?

The percentage of sugar used is 1%

2. What are the indicators used?

The indicators used are bromothymol blue (changes from blue to yellow), andrade's indicator (changes from yellow to pink).

3. What is the purpose of durham's tube?

Durham's tube is to detect the presence of gas.

4. How do you interpret the results?

The fermentation is indicated by the change in the colour of the medium and gas if produced will be present in the durhams tube.

5. How is andrade indicator made?

Andrade's indicator is made by adding NaOH 1mol/litre to a 0.5% solution of acid fuschin until the colour turns yellow. It is used at a final concentration of 0.005% in the medium and it turns dark reddish pink at about pH5.5.

6. Name some lactose fermenting bacteria.

*E.coli, Klebsiella spp. Enterobacter spp, Citrobacter spp.*

7. How do you inoculate sugars?

The sugars are inoculated by stabbing with the straight wire from the liquid media.

### 3.19. Oxidative fermentative test

Bacteria use either carbohydrates or proteins for energy. Determining whether substrate utilization is oxidative or fermentative is used for the identification of different bacteria. Oxidative process require oxygen whereas fermentative process does not. Oxidative fermentative reactions are checked by using semi-solid medium that contains low concentration of protein and one carbohydrate substrate such as glucose.

**Method:** The organism to be identified is inoculated into two tubes. One tube is overlaid with mineral oil(1cm) to create anaerobic environment and the other without oil. The indicators used are either bromocresol purple, phenol red, andrade's indicator and bromothymol blue.

**Results:** When acid reaction is seen in both the tubes it indicates that the organism is fermentative because fermentation can occur with and without oxygen. If acid is detected in only in the open tube, it indicates that the organism is oxidative. The OF test is used to separate organisms which are oxidative (*Pseudomonas group*) and fermentative (Enterobacteriaceae group). Organisms that cannot break down the carbohydrate aerobically or anaerobically (e.g. *Alcaligenes faecalis*) produce an alkaline reaction in the open tube and no change in the covered tube. This medium can also be used for recording gas production and motility.

### Questions

1. Why is OF test done?

OF test is done to detect organisms which are oxidative and which are fermentative.

2. What are the indicators used in OF test?

The indicators used are phenol red (yellow to red), bromothymol blue (yellow to blue) bromocresol purple (purple to yellow), andrade's indicator (yellow to pink).

3. What is the control used for OF test?

*E.coli* ATCC 25922 both oxidative and fermentative.(ie both the tubes with and without oil the indicator changes colour due to utilisation of glucose.

*Pseudomonas aeruginosa* ATCC 27853 is only oxidative (ie only the tube without oil, the indicator changes colour on the top portion of the tube which is exposed to oxygen).

4. What is the colour of positive O/F test?

In our lab we use bromothymol blue as indicator and the colour changes from yellow to blue (acidic to alkaline). When the glucose is utilised the pH drops and the indicator colour changes to yellow.

5. What is the principle of oxidative/fermentative test?

Bacteria use either carbohydrates or proteins for energy. Determining whether substrate utilization is oxidative or fermentative is used for the identification of different bacteria. Oxidative process require oxygen whereas fermentative process does not. Oxidative

fermentative reactions are checked by using semi-solid medium that contains low concentration of protein and one carbohydrate substrate such as glucose.

6. When do you interpret the O/F as oxidative and when as fermentative?

The organism to be identified is inoculated into two tubes. One tube is overlaid with mineral oil (1cm) to create anaerobic environment and the other without oil. The indicators used are either bromocresol purple, phenol red, Andrade's indicator and bromothymol blue.

Interpretation: When acid reaction is seen in both the tubes it indicates that the organism is fermentative because fermentation can occur with and without oxygen. If acid is detected in only in the open tube, it indicates that the organism is oxidative. The OF test is used to separate organisms which are oxidative (*Pseudomonas* group) and fermentative (Enterobacterales group). Organisms that cannot break down the carbohydrate aerobically or anaerobically (e.g. *Alcaligenes faecalis*) produce an alkaline reaction in the open tube and no change in the covered tube. This medium can also be used for recording gas production and motility.

7. Give some examples of bacteria in which O/F test is oxidative.

*Pseudomonas* spp. *Acinetobacter* spp. *Micrococci*.

8. Name some Gram-positive cocci which give oxidative reaction on O/F test.

*Micrococci* is the gram-positive cocci which give oxidative reaction.

### 3.20 Bile esculin test

**Principle:** This test is for the presumptive identification of *Enterococci*. This test helps in differentiating *Enterococci* from other groups of *Streptococci*. *Enterococcus* spp grow in the presence of 4% bile and hydrolyze esculin to esculetin. This in turn reacts with Fe<sup>3+</sup> and forms dark brown to black precipitate.

#### Procedure

Inoculate one to two colonies from the growth on to the agar slant. Incubate at 37 deg and observe for black precipitate.

Quality Control:

Positive control: *Enterococcus faecalis* ATCC 29212

Negative control: *Escherichia coli* ATCC 25922

#### Questions

1. What is the use of bile esculin test?

This test helps in differentiating *Enterococcus spp* from other spp of *Streptococci*

2. What is the principle of bile esculin test?

Gram positive bacteria other than *Streptococci* and *Enterococci* are inhibited by bile salts present in the medium. *Enterococcus spp* grow in the presence of 4% bile and hydrolyze esculin to esculetin. This in turn reacts with  $Fe^{3+}$  and forms black precipitate.

3. What is the positive control and negative control used?

Positive control: *Enterococcus faecalis* ATCC 29212

Negative control: *Escherichia coli* ATCC 25922.

### 3.21. Aminoacid decarboxylase test

These tests are based on the ability of some bacteria to decarboxylate an aminoacid to the corresponding amine with the liberation of carbon dioxide.

**Principle:** The decarboxylase test of Enterobacteriaceae is done with Moller's decarboxylate broth. The production of these decarboxylases is induced by a low pH and as a result of their action, the pH rises to neutrality or above. The low pH(5.5) is necessary initially to enhance the decarboxylase activity. This can be done by the utilization of glucose present in the medium initially to produce mixed acids. Further deamination of aminoacids increases the medium to alkaline pH with the change in colour of indicator to bromocresol purple. The lysine and ornithine reactions are truly decarboxylase tests but the arginine reaction is more correctly recognized now as a dihydrolase test. A control tube devoid of aminoacids should be included to ensure that initial drop in pH has occurred.

The degradation products of the aminoacids are as follows.

Lysine--cadaverine

Ornithine-putrescine

Arginine-citrulline

#### **Procedure:**

The medium contains peptone, meat extract, glucose, pyridoxal, bromocresol purple, distilled water. Dissolve the solids in water and adjust the pH to 6.0 before the addition of the indicators. This is the basal medium and to it is added the amino acid whose decarboxylation is to be tested. Divide the basal medium into four portions and treat separately as follows.

Add 1% L-lysine hydrochloride

Add 1% L-ornithine hydrochloride

Add 1% L-arginine hydrochloride

Control: without addition of amino acid. Adjust the pH to 6.0

Distribute 1ml quantities in small tubes containing sterile liquid paraffin to provide a layer about 5mm thick above the medium. Inoculate lightly through the paraffin layer with a straight wire. Incubate and read daily for 4 days. The medium first becomes yellow due to acid production during glucose fermentation. Later if decarboxylation occurs, the medium becomes violet. The control should remain yellow.

### Questions

1. What is the principle of decarboxylase test?

Answer given in the explanation above.

2. What is the indicator used in decarboxylase test?

The indicator used is bromocresol purple.

3. Mention the positive control and negative control used.

	Positive control	Negative control
Lysine	<i>Klebsiella aerogenes</i>	<i>Klebsiella cloacae</i>
Arginine	<i>Klebsiella cloacae</i>	<i>Klebsiella pneumoniae</i>
Ornithine	<i>Klebsiella cloacae</i>	<i>Klebsiella aerogenes.</i>

4. When do you interpret the test?

The test is interpreted after 4 days.

5. Why do you need to interpret the test after 4 days?

Initially the glucose which is present is utilized to produce acidic environment. The end product of glucose metabolism produce mixed acids. This low pH is necessary for decarboxylation of amino acids which further rises the pH to alkaline. That's the reason the test is read after 4 days.

6. What are the degradation products of aminoacid?

The degradation products of the aminoacids are as follows

Lysine--cadaverine

Ornithine-putrescine

Arginine--- citrulline

7. What is the percentage of aminoacid used?

The percentage of aminoacid used is 1%.

### 3.22. Phenylalanine deamination test

This test is done to differentiate the species of *Providentia*, *Proteus* and *Morganella* group from other species of Enterobacteriaceae family.

**Principle:** This test determines the ability of organism to deaminate phenyl alanine to phenyl pyruvic acid which will react with ferric salts to produce green colour.

Quality control

Positive control: *Proteus spp*

Negative control: *E.coli ATCC 25922*

**Procedure:** After an overnight incubation in PPA tube add few drops of 10% ferric chloride on the sides of the tube. If the test is positive, green colour develops immediately.

### Questions

1. What is the use of PPA test?

This test is done to differentiate *Proteus*, *Providentia*, *Morganella spp* of Enterobacteriaceae from other spp of Enterobacteriaceae family.

2. What is the principle of PPA test?

Answer given in the explanation.

3. What is the positive control and negative control used in PPA test?

Positive control: *Proteus spp*.

Negative control: *Escherichia coli ATCC 25922*

### 3.23. Coagulase test

This test is used to differentiate coagulase positive *S.aureus* and coagulase negative *Staphylococcus spp.*

**Principle:** *Staphylococcus aureus* produce bound coagulase and free coagulase enzyme.

**Slide coagulase test:** Bound coagulase or clumping factor is bound to cell wall and reacts directly with

fibrinogen and results in clumping when mixed with plasma.

**Tube coagulase test.** This test is used for the detection of free coagulase. This reacts with coagulase reacting factor present in plasma and converts fibrinogen to fibrin.

#### **Procedure:**

**Slide coagulase test:** Take a slide. Draw two circles. In one circle emulsify colony and saline which serves as control. In the other circle, take a drop of saline and add a colony and emulsify and then add a drop of undiluted plasma and mix with a wooden applicator stick. Rock the slide back and forth and observe for the prompt clumping of bacterial suspension within 10-15 seconds.

#### **Quality Control:**

Positive control. *Staphylococcus aureus* ATCC 25923(Coagulase positive bacteria)

Negative control. *Staphylococcus epidermidis*. (Coagulase negative bacteria).

**Tube coagulase test:** Take 0.5ml of rabbit plasma (diluted 1 in 5 with saline) in a test tube.

Add approximately 5 drops (250ul) of overnight broth culture or small amount of the colony growth of *S.aureus* to the diluted plasma in the test tube. Incubate the tube at 37deg C for 4 hours. Observe for clot formation by gently tilting the tube.

#### **Questions**

1.How many types of coagulase test do you know?

There are 2 types of coagulase test slide coagulase and tube coagulase.

2.How do you perform slide coagulase test?

The organism must first be cultured on nutrient agar or blood agar

1. Place a drop of distilled water on each end of a slide or on two separate slides.
2. Emulsify a colony of the test organism (previously checked by Gram staining) in each of the drops to make two thick suspensions. Add a loopful (not more) of plasma to one of the suspensions and mix gently. Look for clumping of the organisms within 10 seconds. No plasma is added to the second suspension. This is used to differentiate any granular appearance of the organism from true coagulase.

Positive coagulase control: *Staphylococcus aureus*

Negative coagulase control: *Staphylococcus epidermidis*

3. How do you perform tube coagulase test?

Tube test method (detects free coagulase)

1. Take three small test tubes and label:

T - Test organism (18–24 hr broth culture)

Positive control (18–24 hr *S. aureus* broth culture)

Negative control (sterile broth)

Nutrient broth is suitable.

2. Pipette 0.8 ml of plasma into each tube.

Add 0.2 ml of the test broth culture to tube T. Add 0.8 ml of the plasma to the tube labelled 'Positive'. Keep it in the incubator for 4 hours. If free coagulase is present in culture broth it produces a clot.

3. What is clumping factor?

Clumping factor is bound coagulase present in the cell wall of the bacteria which converts fibrinogen to fibrin. It does not require the presence of coagulase reacting factor.

4. What is the principle of slide coagulase test?

Bound coagulase is also known as clumping factor. It is bound to the bacterial cell wall and is not present in culture filtrates. This reacts with plasma and converts fibrinogen to fibrin.

5. What is the principle of tube coagulase test?

Free coagulase is an extracellular enzyme produced by *Staphylococcus aureus*. It reacts with coagulase reacting factor present in plasma and converts fibrinogen to fibrin.

There are 8 antigenic types of coagulase. Most human strains of *S. aureus* produce coagulase A

6. What is the positive control in the test?

The positive control is coagulase positive *S. aureus*.

7. What is the negative control in the test?

The negative control is coagulase negative *S. epidermidis*

8. How will you interpret slide coagulase test?

Interpretation is done by looking for agglutination.

9. How will you interpret tube?

Look for clot formation after 4 hrs of incubation.

10. What happens to the clot on prolonged incubation?

The clot will be lysed by fibrinolysin produced by the organism.

11. Can you use citrated plasma for the coagulase test?

Citrated plasma should not be used as some bacteria esp. *Pseudomonas spp.* will utilise citrate and gives false positive result.

12. Give some examples of coagulase positive bacteria.

*S. aureus*

*S. schleiferi*

*S. felis*

*S. lutrae*

*S. intermedius*

*S. hyicus*

*S. lugdunensis*

*Peptostreptococcus hydrogenalis*

### **3.24 Bacteriology Questions related to common organisms**

***Staph aureus* (mixed culture)**

1. What are the points in favour of your presumptive diagnosis as *S.aureus*?

It is catalase positive and on gram stain gram positive cocci in clusters, yellow colony on nutrient agar and mannitol fermenting on mannitol salt agar. Both slide and tube coagulase test is positive.

2. Name one test which differentiates genus *Staphylococcus spp.* from *Streptococcus spp.*

Catalase test helps to differentiate *Staphylococcus spp* from *Streptococcus spp.*

*Staphylococcus* is catalase positive whereas *Streptococcus* is catalase negative.

3 How will you differentiate *Staphylococcus* from *Micrococcus*?

Property	<i>Staphylococcus</i>	<i>Micrococcus</i>
Catalase	positive	positive
Micro oxidase test	negative	positive
Bacitracin test	resistant	susceptible
Lysostaphin	susceptible	resistant
Carbohydrate utilisation	fermentative	oxidative

4. Name some toxins produced by *Staph.aureus*.

Hemolytic toxins, enterotoxin, epidermolytic toxin, toxic shock syndrome toxin 1 and 2.

5. Name the enzymes produced by *S.aureus*

Coagulase enzymes, heat stable thermonuclease, DNase, hyaluronidase, lipases, proteases, phosphatase.

6. List the diseases produced by *S.aureus*.

Skin and soft tissue infections, food poisoning, musculo skeletal infections, UTI, meningitis, respiratory tract infection, bacteraemia, Staphylococcal scalded syndrome.

7. Name the toxin responsible for Staphylococcal food poisoning.

Enterotoxin is responsible for food poisoning.

8. How many different types of enterotoxins of *S.aureus* are there?

Enterotoxin is typed into A-E, G-I, R-T and V. Type A being more common.

9. Which toxin is responsible for scalded skin syndrome?

Exfoliative /epidermolytic toxin is responsible Staphylococcal scalded syndrome.

10. Which toxin is responsible for toxic shock syndrome?

Toxic shock syndrome toxin 1 and Toxic shock syndrome toxin 2 is responsible for toxic shock syndrome.

11. Name one selective medium used for isolating *Staph. aureus*.

Mannitol salt agar is the selective medium for *Staph. aureus*

12. In which bacteria mannitol fermentation is an important test for diagnosis or for some other characteristics?

In *Staphylococcus aureus*, mannitol fermentation is important as it's the only spp. of *S. aureus* which will ferment mannitol.

13. What typing method is employed for *Staph. aureus*?

Bacteriophage typing method is employed for *Staph aureus*.

14. What is the use of typing method?

The typing method is used for epidemiological study to trace the source of infection.

15. How many phages are used for bacteriophage typing of *S. aureus*?

Phages 80 and 81 is associated with outbreaks in hospitals.

16. Which gene is responsible for Methicillin resistant *Staphylococcus aureus* (MRSA)?

Mec A gene is responsible for MRSA.

### ***Enterococcus spp.***

1. Name few enterococci

*Enterococcus faecalis*, *E. faecium*, *Enterococcus durans*, *Enterococcus avium*, *Enterococcus casseliflavus*.

2. How can *E. faecalis* be identified?

*E. faecalis* ferments mannitol, ferments pyruvate, raffinose not fermented and sorbitol fermented, bile esculin is hydrolysed.

3. What are the tests used to identify *Enterococcus faecalis*?

Production of NH<sub>3</sub> from arginine, aesculin hydrolysis and positive pyrrolidinyl peptidase and fermentation of mannitol, pyruvate and sorbitol helps identification of *Enterococcus faecalis*.

4. What is heat resistance test?

Heat resistance test is the test done to differentiate Group D Streptococci from Enterococcus.

5. How do you perform heat resistance test?

It is performed by inoculating organism in peptone water and keeping it in water bath at 60 deg for 30 min. After that the organism is inoculated on nutrient agar. If the growth occurs, it indicates it is resistant to heat.

6. Heat resistance test is useful for which bacteria?

Heat resistance test is useful to differentiate *Enterococcus spp* from Group D Streptococci.

7. How will you confirm group D streptococci?

The group D streptococci differ from enterococci in that it does not grow in 6.5% NaCl, does not form NH<sub>3</sub> from arginine, does not survive at 60 deg for 30 min.

8. How do you differentiate group D *Streptococci* from *Enterococcus*?

*Enterococcus* is differentiated by production of acetoin, pyrrolidinyl peptidase, ability to grow in broth containing 6.5% NaCl and fermentation of esculin on nutrient agar with 4% bile.

9. Name infections caused by *E. faecalis*

Urinary tract infection, biliary tract infections, suppurative abdominal lesions and endocarditis.

10. Name the drug used for penicillin resistant strains of *E. faecalis*

Vancomycin is the drug used for penicillin resistant strains of *E. faecalis*.

### ***Vibrio cholerae***

1. Name the different media used for growing *Vibrio cholerae*.

TCBS, Bile salt agar, Monsur's gelatin taurocholate trypticase tellurite agar.

2. How do you transport stool sample to the laboratory?

Cary blair medium or alkaline peptone water are used for transporting stool sample to lab when you suspect *Vibrio cholerae*.

3. What are halophilic vibrios?

Halophilic vibrios are vibrios which can tolerate >6% salt concentration whereas Vibrios can tolerate <6% salt concentration.

4. How do you differentiate *Vibrio cholerae* from halophilic vibrios?

Halophilic vibrios are vibrios which can tolerate >6% salt concentration whereas Vibrios can tolerate <6% salt concentration.

5. What are non-agglutinating vibrios?

Serogroups other than O1 vibrios which are not agglutinated by O1 antiserum are called non-agglutinable vibrios.

6. How do you differentiate classical and Eltor *Vibrio cholerae*?

Biotypes of <i>Vibrio cholerae</i> O1	Classical	Eltor
B hemolysis on blood agar	Negative	Positive
Chick erythrocyte agglutination	Negative	positive
Cholera toxin gene	CTX 1	CTX 2
Polymyxin B 50 units	susceptible	resistant
VP (Voges-Proskauer test)	negative	positive
Eltor phage V susceptibility	resistant	susceptible
Group IV phage susceptibility	susceptible	resistant

7. What is cholera red reaction?

Indole and nitrate reduction properties can be tested together by adding few drops of sulfuric acid to a peptone water culture of *Vibrio cholerae*. A reddish pink color nitroso-indole ring is formed.

8. How many O serotypes of *Vibrio cholerae* are there?

Serogroups are divided into 3 serotypes which include Ogawa, Inaba and Hijojima.

9. How many pandemics of cholera have occurred till today?

First six pandemics occurred between 1817 and 1923. All were caused by the classical biotype of *V. cholerae* which had spread from Bengal to involve most of the world. After the end of the 6th pandemic, from 1923 to 1961 cholera was largely restricted to its home land. Seventh pandemic was the only pandemic that originated outside India, i.e. from Indonesia (Sulawesi, formerly Celebes Island) in 1961. It was the only pandemic to be caused by El

Tor biotype. El Tor infection was associated with more carrier rate than the classical. This is due to the fact that El Tor is much harder than the classical vibrios and capable of surviving in the environment much longer.

10. Which *Vibrio cholerae* is responsible for current pandemic going on?

*Eltor vibrio* is responsible for current pandemic.

11. What are the bio types and serotypes of *V. cholerae*?

Biotypes are Classical and Eltor.

Serotypes include Ogawa, Inaba and Hikojima.

12. How do you do slide agglutination for identification for identification of *V. cholerae*?

Slide agglutination is done by emulsifying the colony of *V. cholerae* from the culture plate and mixed with *V. cholerae* polyvalent O antiserum. Serogrouping is done with O1 specific antisera. Once agglutination is done biotyping is done with Ogawa and Inaba antisera.

13. What are the colony characteristics of *V. cholerae* on different selective media?

**TCBS agar**- Colonies are yellow due to fermentation of sucrose.

**Bile salt agar**- Colonies are translucent, glistening oil drop.

**Mansur's taurocholate tellurite agar**- Translucent colonies with greyish black centre and a turbid halo

14. Where is the national reference centre for *Vibrio cholerae* located in India?

National reference Center for cholera in India is located at National Institute of Cholera and Enteric Diseases (NICED), Kolkata.

### ***Salmonella spp.***

1. What are the points in favour of your presumptive diagnosis of *Salmonella spp*?

Non lactose fermenting colonies on Macconkey agar, motile Gram-negative bacilli, catalase positive, oxidase negative, Green colonies with black centre on WBBS, Indole negative TSI K/A with gas -ve, H<sub>2</sub>S specks. Urease not hydrolysed, citrate not utilised, and agglutination with poly O antiserum and factor 9 in favour of *S. Typhi*.

2. Name the selective media for *Salmonella typhi*.

WBBS (Wilson and Blair bismuth bisulphite), Salmonella Shigella agar.

3. Name some non-motile *Salmonella spp.*

*Salmonella gallinarum* and *S.pullorum* are non-motile salmonella.

4.Mention the enrichment media used for Salmonella.

Selenite F broth and also Gram-negative broth are the enrichment medias used for Salmonella.

5.How would you differentiate various species of Salmonella?

S.Typhi.Indole negative, TSI K/A Gas -ve, H<sub>2</sub>S specks. Agglutination with Factor 9

S.Paratyphi A indole negative, TSI K/A gas +ve,H<sub>2</sub>S negative, Agglutination with factor 2

S.Paratyphi B indole negative, TSI K/A gas +ve, H<sub>2</sub>S abundant agglutination with Factor 4

6.Name the Salmonellae causing enteric fever.

Enteric fever is caused by any species of Salmonella. Typhoid fever is caused by *S.Typhi*.

7.Name the diseases (other than enteric fever) caused by Salmonellae.

Gastroenteritis, septicaemia, focal infections like osteomyelitis, meningitis, endocarditis, brain abscess.

8.Name lactose fermenting *Salmonella spp.*

*Salmonella virchow* is lactose fermenting bacilli.

9.Name the various specimens used for laboratory diagnosis of enteric fever.

Blood, stool, urine, Bone marrow, Duodenal aspirate, sewage sample.

10. What is clot culture?

When the blood is centrifuged the serum is separated and used for widal test whereas the clot is used for clot culture test. The clot is lysed with the help of streptolysin. Clot culture has shown a higher isolation rate than blood culture as the bactericidal action of serum is not there.

11. What is widal test?

Widal test is a serological test for the diagnosis of typhoid fever. It is an agglutination test where H and O antibodies against *S. Typhi*, *S. Paratyphi A* and *B* are detected and measured in the patient's sera by using O and H antigens.

12. What is anamnestic reaction?

It refers to a transient rise of widal titre due to unrelated infections (malaria, dengue) in persons who have had prior enteric fever.

13. Name the various tests to diagnose carrier state in typhoid fever.

By stool and bile culture (detects fecal carriers) and urine culture (detects urinary carriers)

14. Where is the National Salmonella phage typing centre situated in India?

National Salmonella Phage Typing Center-at Lady Harding medical college, New Delhi

National Salmonella Reference Centre-at Central Research Institute, Kasauli

National Salmonella Reference Centre for animal origin-at Izatnagar.

15. How does *Salmonella typhi* acquire multiple drug resistance?

Multidrug resistance (MDR) of *Salmonella typhi* is plasmid mediated due to mutations in the *gyrA*

16. What is the route of administration of Typhoid vaccine ?

Parenteral Vi polysaccharide vaccine derived from *S. Typhi* strain Ty2 is given IM or subcutaneously and the booster dose is given every 2 years.

17. Name the newer vaccines available for typhoid fever.

Vi polysaccharide vaccine is available which is given as subcutaneous administration.

18. Name the Salmonellae which cause gastroenteritis.

*S. typhimurium* and *S. enteritidis*, *S. newport*, *S. javiana*, *S. heidelberg*, *S. choleraesuis* and *S. dublin*.

19. Name non-lactose fermenter bacteria which are motile.

Proteus group are non lactose fermenter group which are motile

20. List gram negative bacilli which are motile.

*Escherichia coli*, *Citrobacter* group, *Salmonellae* group, *Proteaceae* group.

21. Name some Gram-positive bacilli.

*Clostridium* spp, *Bacillus anthracis*.

### ***Shigella* spp.**

1. What are the points in favour of your presumptive diagnosis as *Shigella* spp?

They are gram negative bacilli, catalase positive and oxidase negative, non-motile and nonlactose fermenting colonies on MacConkey agar, indole negative, TSI K/A anaerogenic and H<sub>2</sub>S negative, urease negative, citrate negative. Agglutination with *Shigella* antisera occurs.

2. How will you diagnose the different species of *Shigella*?

1. *Shigella dysenteriae* has got 15 serotypes. *Shigella dysenteriae* type 1 is catalase negative,

*S. dysenteriae* (group A): It has 15 serotypes. It does not ferment mannitol in contrast to other species of *Shigella*, which are mannitol fermenters.

Serotype 1 (*S. shigae*) is the only *Shigella*, which produces Shiga toxin. It is the only member of family Enterobacteriaceae to be catalase negative. It is indole negative.

Serotype 2 (*S. schmitzi*) forms indole and ferments sorbitol and rhamnose.

Serotype 3-7 were formerly called Large-Sachs group.

2. *S. flexneri* (group B): Based on type specific antigen, it is further typed into 8 serotypes.

*S. flexneri* is the most complex species antigenically.

Serotype 8 is always indole negative and is further typed to three biotypes-Manchester, Newcastle and Boyd 88 based on gas production from sugars.

*S. flexneri* can also be typed by bacteriophage typing into 123 phage types.

*S. boydii* (group C): It has 19 serotypes. It was first described by Boyd. *S. boydii* is isolated least frequently from cases of bacillary dysentery.

*S. sonnei* (group D): It is antigenically homogeneous and has only one serotype. It can be typed by colicin typing into 26 serotypes.

3. How will you confirm the isolated bacteria as *Shigella spp*?

Confirmation is done by agglutination with *Shigella* antisera.

4. Name the enrichment medium used for *Shigella spp*.

Selenite F broth, tetrathionate broth and GN broth are the enrichment medium used for *Shigella spp*.

5. Name some solid culture media used to grow *Shigella*.

XLD (xylose lysine deoxycholate) and DCA (Deoxycholate citrate agar) and Salmonella shigella agar are the selective media to grow *Shigella*.

6. Classify *Shigella* species

*Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei*.

7. How many serotypes are those in each *Shigella spp*?

Ans explained in question 2.

8. Name the *Shigellae* which produce acid and gas on fermentation of carbohydrates.

(*S. flexneri* serotype 6 and *S. boydii* serotype (14) ferments carbohydrates and produce gas.

9. Name lactose fermenter *Shigella*.

*Shigella sonnei* is late lactose fermenter.

10. What is Large-Sachs group?

*Shigella dysenteriae* Serotype 3-7 are called Large-Sachs group.

11. Name the typing methods used for *shigella sonnei*.

Bacteriocin and colicin typing are the typing methods done for *Shigella sonnei*.

12. Which *Shigella spp.* causes the most severe forms of dysentery?

*Shigella dysenteriae* causes the most severe forms of dysentery.

13. Name the toxins produced by *Shigella gp.*

*Shigella* enterotoxins 1 and 2, shiga toxin and endotoxin.

14. Name the typing methods used for *Shigella sonnei*.

Bacteriocin and colicin typing are the typing methods done for *Shigella sonnei*.

15. Which *Shigella spp.* causes the most severe forms of dysentery?

*Shigella dysenteriae* causes the most severe forms of dysentery.

16. What is the mode of infection in bacillary dysentery and what is the load of the bacilli to cause infection?

The mode of entry is thorough contamination of water with faeces and the load of the bacilli is 10-100 bacilli.

17. Which *Shigella spp* produces toxemia?

*Shigella dysenteriae* produces toxemia producing shiga toxin.

## **E.coli**

1. What are the points in favour of presumptive diagnosis as *E. coli*?

Gram negative bacilli, catalase positive, oxidase negative, motile with lactose fermenting colonies on macconkey agar, Indole positive TSI A/A gas + H<sub>2</sub>S -ve. urease not hydrolysed, citrate not utilised and nitrates reduced to nitrites are in favour of *E.coli*.

2. Which antigens are involved in serotyping of *Escherichia coli*?

O and H antigens are involved in serotyping.

3. What are the characteristics of the members of family Enterobacteriaceae?

They are gram negative bacilli which are catalase positive, oxidase negative, motile or non-motile, ferments glucose with or without gas and reduces nitrates to nitrites.

4. Describe the mode of action of LT.

Enterotoxigenic *E. coli*. It resembles cholera toxin in its structure and function, but it is less potent than the latter. It has 2 peptide fragments: A and B

Fragment B: It is the binding fragment, has five subunits. It binds to GM1 ganglioside receptors present on the intestinal epithelium following which A fragment is internalized and cleaved into A1 and A2 peptides.

Fragment A. Fragment A2 helps in tethering A and B subunits together.

Fragment A1 is the active fragment (27 kDa), causes ADP ribosylation of G protein and upregulates the activity of adenylate cyclase. This results in the intracellular accumulation of cyclic AMP (adenosine monophosphate) which leads to increased outflow of water and electrolytes into the gut lumen, with consequent diarrhoea.

5. Describe the mode of action of ST.

Enterotoxigenic *E. coli* ST is of two types: ST-I and ST-II.

Mechanism of action: ST-I: Binds to the guanylate cyclase C which causes increased production of cyclic guanosine monophosphate (cGMP). This causes fluid accumulation in gut lumen resulting in diarrhoea. ST-II: Though it causes fluid accumulation (in young piglets), the mechanism is not known yet, it is not through cAMP or cGMP mediated.

6. Name the diseases caused by *Escherichia coli*.

UTI, diarrhoea, meningeal infections.

7. What is Sereny test?

In case of Entero invasive *E. coli*, (inoculation of bacterial suspension into guinea pig eyes produces conjunctivitis. This is called as sereny test.

8. Name the conditions or syndrome caused by EHEC.

Haemorrhagic syndrome is caused by EHEC.

9. What is alkalescens-dispar group?

It is *E.coli* inactive form which is non motile and does not ferment lactose and anaerogenic.

10.Name the enterotoxin produced by EAEC.

EAST ( Entero aggregative heat stable toxin is produced by EAEC)

**3.25. Betalactamases**

1. What are beta lactamases?

$\beta$ -lactamase enzymes are enzymes produced by the bacteria which are capable of hydrolyzing the  $\beta$ lactam rings (the active site) of  $\beta$ -lactam antibiotics. They can be produced by both gram-positive and gram-negative organisms. They are plasmid coded, and transferred from one bacterium to other mostly by conjugation, (except in *Staphylococcus aureus* where they are transferred by transduction).

2.Classify Beta lactamases.

Beta lactamases can be classified in two ways.

Ambler’s classification. Structural or molecular classification. According to this beta lactamases are classified into 4 classes.

Bush Jacoby Medeiros classification- most advanced and complex classification.

Ambler’s classification

Class	Active site	Characteristics	example
A	Serine	Broad-spectrum enzymes, including most ESBLs and serine carbapenemases	TEM-1, SHV-1, KPC, OXY, CTX-M
B	zincs	Metallo- $\beta$ -lactamases hydrolyzing most $\beta$ -lactams, including carbapenems but not monobactams	VIM, IMP, SPM, NDM
C	serine	Broad-spectrum enzymes hydrolyzing cephalosporin subclasses III and IV and cephamycins	AmpC, ACT, CMY, PDC
D	serine	Oxacillin-hydrolyzing enzymes	OXA (including carbapenemase phenotypes and rare ESBLs)

3. What are ESBLs?

Extended spectrum beta lactamases are class A and Class D enzymes that hydrolyse cephalosporins and monobactams and are inhibited by clavulanate. The resistance can be overcome by the use of  $\beta$ -lactam along with  $\beta$ -lactamase inhibitor (e.g. sulbactam or clavulanate). The genes encoding  $\beta$ -lactamases may be located on chromosomes and expressed with or without induction or carried on plasmids in single or multiple copies.

4. What are ampC enzymes?

AmpC beta-lactamase enzyme producers are resistant to cephamycin (e.g.cefoxitin and cefotetan). They can be either chromosomal mediated or plasmid mediated. But they are

sensitive to carbapenems. Resistance cannot be overcome by  $\beta$ -lactam +  $\beta$ -lactamase inhibitor combination (BL/BLI).

5. What are carbapenamase enzymes?

Carbapenamases are enzymes produced by the bacteria which are resistant to carbapenems group of drugs. Their resistance cannot be overcome by BL/BLI. The mechanism of resistance could be due to production of carbapenamase enzymes, impermeability due to porin mechanism. Important carbapenamase enzymes are: *Klebsiella pneumoniae* carbapenamase (KPC), New Delhi metallo-beta-lactamase (NDM) and OXA enzymes. NDM-type and other metallo- $\beta$ -lactamase enzymes need zinc for activity and are inhibited by substances that bind zinc (eg, EDTA).

6. How do you detect ESBL in vitro?

This can be detected by disc diffusion method using. Combined Disk tests (Cefotaxime + Cefotaxime clavulanate, Ceftazidime + Ceftazidime clavulanate (Zone of Inhibition of Ceftazidime clavulanate >5mm than Zone of Inhibition of Ceftazidime) indicates ESBL production.

7. How do you detect Ampc beta lactamase?

This can be detected by using two discs cefoxitin which is a strong inducer and cefotaxime weak inducer. The strong inducer antagonises the action of weak inducer. The discs are placed centre to centre by disc diffusion method. If amp C beta lactamase is present, we can see no zone around cefoxitin and there will be flattening on cefotaxime disc facing cefoxitin side.

8. How do you detect carbapenamases?

This can be detected by m-cim test, carb NP test e-cim method. Earlier modified Hodge test was done which has become obsolete now.

9. What are BL/BLI?

BLI are betalactamase inhibitors are drugs which inactivates the beta lactamase enzymes. These include clavulanate, sulbactam and tazobactam.

Eg of drugs include Ceftazidime/Ceftazidime clavulanic acid, Cefperazone and cefperazone/sulbactam, Piperacillin and piperacillin tazobactam

9. What is inducible clindamycin resistance?

Macrolide-resistant isolates of *S. aureus*, CONS, *S. pneumoniae*, and  $\beta$ -hemolytic streptococci may express constitutive or inducible resistance to clindamycin (23S ribosomal RNA methylation encoded by the erm gene, also referred to as MLSB [macrolide, lincosamide, and type B streptogramin] resistance) or may be resistant only to macrolides (efflux mechanism encoded by the msr(A) gene in staphylococci or a mef gene in streptococci).

10. How do you perform the inducible clindamycin resistance?

The disk diffusion test is performed by placing a 2- $\mu$ g clindamycin disk either 15 mm away (for staphylococci) or 12 mm away (for streptococci) from the edge of a 15- $\mu$ g erythromycin disk on either of the following media. Flattening of the clindamycin zone adjacent to the erythromycin disk (referred to as a D-zone) indicates inducible clindamycin resistance. Constitutively resistance strains grow up to both the discs whereas inducible resistance there is a zone around the clindamycin which is flattened on the side adjacent to erythromycin.

## 4.MYCOLOGY



#### 4.1. Collection of specimens

**Superficial mycoses:** The specimens for fungal examinations are generally collected in folded squares of black paper. The purpose is to permit drying of specimen, prevent bacterial contamination and to preserve samples for a longer duration without appreciable loss to viability of fungal agents. The dermatophytic lesions usually spread outward in a concentric fashion with healing in central region. Therefore, material should be collected by scraping outward from edges of lesion with scalpel blade held at an angle of 90 deg to skin surface. Hair should be plucked from scalp with the help of forceps. The hairbrush sampling can be utilized to obtain specimens for fungal cultures which can be done by thoroughly brushing scalp and using hairbrush to inoculate onto culture media. Nail should be taken from discoloured part of the nail. We need to collect full thickness of the nail and as far back as possible from the edge of the nail.

**Subcutaneous mycoses:** The scrapings or crusts from superficial parts of subcutaneous lesions is enough for culture.

**Systemic mycoses:** Here generally samples like pus, faeces, sputum, spinal fluid, blood and scrapings or swabs from the edge of the lesions are considered. Most of the specimens can be examined in wet mount after partial digestion with 10-20% KOH.

#### Questions

1. What are the different ways of collecting the samples for fungal examination?

Answer explained above.

#### 4.2. KOH stain

KOH stain is the preliminary step for any samples that come for fungal examination.

10% KOH is used for hair, tissues, skin scrapings and 40% KOH is used for nail clippings or any sample which is difficult to dissolve.

**Principle:** KOH may be used to examine hair, nails, skin scrapings, fluids, exudates, or biopsies. KOH helps in dissolving human cellular debris and helps in easier visualization of fungal elements.

Specimens placed in a drop of 10% KOH will dissolve at a greater rate than fungi because fungi have chitinous cell walls. The clearing effect throughout the clinical specimen can be accelerated by gently heating the KOH preparation.

## **Procedure**

Emulsify the specimen in a drop of 10% KOH on a microscopic slide with the help of a loop. Apply gentle heat by passing the slide over a bunsen flame for 3 to 4 times.

Cover the smear with the cover slip. Leave it for 5-10 minutes.

Examine the slide under low(10X) and high power (40X) magnifications.

Examine the slide for 15-20 minutes for demonstration of shining fungal elements.

## **Results and interpretations**

Different fungi will have different morphological forms (yeast cells with pseudo hyphae, budding septate

and aseptate hyphae, granules, etc.) which can be clearly seen in a KOH wet mount.

Interpretation of results should be done by critical analysis of the type, size and color of fungal elements which will be different for different fungi.

## **Questions**

1.What is the percentage of KOH used?

We use 10% KOH for hair, skin scrapings, 40% KOH for nail clippings.

2.What is the function of KOH?

The function of KOH is to clear out the background scales or cell membranes that may be confused with fungal hyphal elements in microscopy of clinical specimens.

3.How do you interpret results of KOH?

Different fungi will have different morphological forms (yeast cells with pseudo hyphae, budding septate and aseptate hyphae, granules, etc.) which can be clearly seen in a KOH wet mount.

Interpretation of results should be done by critical analysis of the type, size and color of fungal elements which will be different for different fungi

4.How do you store KOH?

KOH bottles should be stored in ambered colour bottles and also loses its efficacy if stored for longer time. It is advisable to prepare fresh ones.

5. What are the other methods of demonstration of fungal elements?

The other methods of demonstration of fungal elements are

- Gram stain for demonstration of yeast eg. *Candida spp.* and *Cryptococcus spp.*
- India ink and nigrosin – demonstration of capsulated fungi.
- Vinyl Adhesive tape (scotch tape) technique for demonstration of fungi like *Malassezia spp.*, *Candida spp.* and dermatophytes.
- Hematoxylin & Eosin stain is a routine procedure in histopathology section. Other stains include Periodic Acid Schiff (PAS) stain, Grocott Gomori Methenamine Silver (GMS) stain, Gridley stain to demonstrate fungal elements in tissues.
- Mayer's mucicarmine stain—to demonstrate capsular material of *Cryptococcus spp.* and sporangia of *Rhinosporidium seeberi*.
- Masson-fontana stain to demonstrate phaeoid fungi in tissues.
- Fluorescent stain—calco fluor white stain can bind to cellulose and chitin of fungal cell wall and visualised when exposed to long wavelength and ultraviolet and short wavelength visible light.

#### 4.3. Gram stain for fungi

Gram stain is done mainly for the staining of *Candida spp.* The procedure is same as Gram stain explained in bacteriology section.

The *Candida spp.* looks like gram positive budding yeast cells.

#### 4.4 India ink test – demonstration of capsule

**Principle:** India ink is used as a negative stain preparation. When used in wet mount preparation of the CSF, the background appears black, and the unstained capsule of *Cryptococcus neoformans* appears as a white halo around the yeast cells in microscopy.

#### **Procedure**

1. Put a drop of CSF on the microscopic slide.
2. Put a drop of India ink to the CSF on the microscopic slide.
3. Emulsify the specimen with India ink on the slide.
4. Place a cover slip over the preparation, taking care not to trap air bubbles in the preparation

5. Blot dry the excess fluid.
6. Examine the slide under low (10X) and high power (40X).

**Results:** India ink stains the background and leaves a clear halo around the cell. Usually the background is stained and the capsule is unstained. Mainly used for identification of *Cryptococcus neoformans*.

### Questions

1. What is the percentage of India ink used?

The percentage of India ink used is 0.5%.

2. What is the advantage of India ink preparation and how is it useful in emergency mycology laboratory?

India ink preparation is one of the best methods to demonstrate the presence of capsule in case of capsulated yeasts. The demonstration of capsulated yeast like *C. neoformans* in CSF is significant in emergency mycology laboratory for prompt administration of antifungal agents.

3. How to interpret results of India ink preparation?

The India ink preparation should be critically interpreted for the presence of a clear capsule around the yeast cell. This should not be confused with any air bubble that might be present.

4. Is there any other stain to demonstrate capsule? Which is better?

Nigrosin is used for demonstration of capsule. It is a better stain compared to India ink as no carbon particles appear in stain solution and it can last for more than one year.

### 4.5. Lactophenol cotton blue preparation (LPCB)

Lactophenol cotton blue is the most used method adopted in mycology laboratory to identify filamentous fungi.

#### Principle:

Identification of filamentous fungi is made by their characteristic microscopic morphology such as shape, size, arrangement of spore and hyphae. LPCB can be used for demonstration of fungal elements in

- a.Scotch tape preparation
- b.Tease mount preparation
- c.Slide culture preparation

**Scotch tape preparation:** The fungal elements are undisturbed. Touch the adhesive tape of small length on the surface of the colony. Take a drop of LPCB on a slide and place the tape with the colony on the side and examine under microscope.

**Tease mount preparation:** The fungal elements are disturbed but finer details of the fungus can be visualized. Place a small drop of LPCB on a slide. Take the colony with a teasing needle that is neither in the centre nor at the edge.

With other needle tease it properly so that it forms a thin preparation. Place a cover slip and examine under microscope.

**Slide culture:** It gives clear view of undisturbed morphology of fungus.

LPCB ingredients contains phenol crystals, lactic acid, glycerol and distilled water and dye cotton blue.

### Questions

1. What is LPCB and role of its constituents?

LPCB ingredients contains phenol crystals, lactic acid, glycerol and distilled water and dye cotton blue.

Lactic acid helps in preserving the morphology of the fungal element.

Phenol acts as disinfectant.

Cotton blue stains the fungal element.

Glycerol hygroscopic agent prevents drying.

2. What is the other use of LPCB apart from examination of fungi?

LPCB is also used in wet mount of stool for intestinal parasites.

3. What is the advantage of slide culture?

Answer given in the explanation above.

4. What is the advantage scotch tape technique?

Scotchtape technique is used to see fungal morphology without teasing them. The fungal elements are not disturbed. Mainly used for identification of *Malassesia*, subcutaneous fungal infections like chromoblastomycosis, lobomycosis, cutaneous manifestations of systemic infections like paracoccidioidomycosis and dermatophytes.

5. What is the disadvantage of tease mount preparation?

Here the fungal elements are disturbed.

#### **4.6. Fungal culture**

The optimum temperature for most of the fungal growth is 25 to 30 deg C especially dermatophytes which form molds at 25 to 30 deg C and yeast at 37 deg C. You should have knowledge of different fungal medias used. The commonly used media for culture of fungus are

**Sabouraud's dextrose agar:** Sabouraud dextrose agar may be supplemented with antibiotics such as gentamicin and chloramphenicol to minimize bacterial contamination and cycloheximide to inhibit saprotrophic fungi.

**Composition:** 40gm of glucose, 10gm of neopeptone and 35 gm of agar in 1000ml of distilled water. pH is 5.5-6.0. The slants are preferred as compared to plates for isolation of dimorphic fungi as they are safer, require less space and are more resistant to desiccation during protracted incubation.

#### **List of medias used for culture**

Sabouraud's dextrose agar

Sabouraud's dextrose agar with antibiotics

Potato dextrose agar

Corn meal agar

Czapek-dox medium

Gelatin medium

Bird seed agar

Trypticase soy agar

Rice agar with tween 20

Dermatophyte test medium

## Questions

1. List some fungal medias.

Answer explained above.

2. What is the pH of SDA?

The pH of SDA is 5.5-6.0.

3. What is the percentage of glucose used in SDA?

4% of glucose is used in SDA.

4. Which is the media used for isolation of *Cryptococcus spp*?

Bird seed agar is the media used for isolation of *Cryptococcus spp*.

5. Mention the nutritionally deficient medias?

Nutritionally deficient media include corn meal agar, rice starch agar. They are low in nutrients. With the result they suppress vegetative growth and stimulate sporulation in fungi.

6. Which media is used to show the sporulation better?

Potato dextrose agar is used for sporulation as potato and dextrose present in the medium promote luxuriant growth.

### 4.7. Slide culture

Slide culture is a very useful technique to study undisturbed morphological details of fungi especially in finding out the relationship between reproductive structures like conidia. The entire fungal colony can be demonstrated within a short period of time with the use of minimum materials.

#### Procedure:

1. From the petridish containing SDA cut one square cm block of agar for each slide culture to be inoculated.
2. The block can be cut 1X1cm with a help of a sterile coverslip or with the help of sterile test tube that has no lip. Cut a small piece of agar that has a depth of 2mm.
3. Take a sterile slide and place the agar block at the centre. In a petridish keep two sterile rods at an angle and keep a sterile cotton which is wetted with distilled water to provide moisture.

4. Keep the slide with agar on top of the sterile rods and then inoculate 4 corners with the fungal elements. Place a sterile cover slip and press gently. Incubate at 35 deg C. After two days of incubation gently remove the slide and place it on slide with small drop of LPCB. Examine under microscope. You can see undisturbed view of fungal elements.

5. Usually a minimum of 48 hrs is needed before a slide culture shows growth of aerial hyphae. So the results should be interpreted after 48 hours.

### **Questions**

1. What is the advantage of slide culture?

Slide culture gives the undisturbed morphology of fungal colony particularly relationship between reproductive structures like conidia, conidiophore and hyphae. It is used to study an entire fungal colony within a short period of time with the use of minimum materials.

### **4.8. Germ tube test**

**Principle:** Germ tube is an initial hypha from a sprouting conidia, spore or yeast. Formation of germ tube can be demonstrated by inoculating serum with a small quantity of growth of *Candida spp.*

The suspension is incubated at 37 deg C for a minimum of 1 and half hours, after which a drop is examined under the microscope for demonstration of germ tube. This is also known as Reynolds- Braude phenomenon.

### **Procedure**

1. Take three test tubes and label them as 1, 2 and 3.

2. Add 0.5 ml of serum to each of the test tube.

3. Take half of a single colony to be tested by using a sterile loop and mix with serum in the test tube 1.

4. Take half of *C.albicans* single colony and mix it with serum in the test tube 2.

5. Take half of *C.parapsilosis* single colony and mix it with serum in the test tube 3.

6. Incubate at 37 deg C for maximum 2hrs.

7. Place a drop from all the tubes on a slide, cover it with cover slip and examine under 40X objective.

## Quality Control:

Positive control: *Candida albicans*

Negative control: *Candida parapsilosis*

Uninoculated tube should be checked for contamination prior to use.

Germ tubes are seen as long tube-like projections extending from yeast cells.

## Questions

1. When do you do germ tube test?

This test is done for the presumptive identification of *Candida albicans* from clinical samples.

2. What is the other name for Germ tube test?

It is also known as Reynolds- Braude phenomenon.

3. How is the test done?

The procedure is explained above.

4. How do you differentiate true hyphae from pseudo hyphae?

Germ tube	Pseudo hyphae
No constriction at the site of attachment	Constrictions at the site of attachment
Non septate with parallel sides	Septate and not necessarily with parallel sides

5. When do you interpret the test and why?

The test is interpreted after 2 hours. The germ tubes are seen as long tube-like projections extending from yeast cell. They grow at distal end and there is no constriction at the point of attachment to yeast cell. The preparation should not be interpreted after 4 hours as other fungi which produce hyphae begin to germinate beyond this time.

6. Give some examples of fungi where germ tube is positive?

Germ tube is positive in *Candida albicans*, *Candida dubliniensis*. It is only presumptive identification of *Candida albicans* and *Candida dubliniensis*.

7. How do you differentiate *Candida albicans* and *Candida dubliniensis*?

These two can be differentiated based on chlamydospores formation. *Candida dubliniensis* produce more chlamydospores than *Candida albicans*.

#### 4.9 Hair perforation test

This test is done to differentiate between dermatophytes *T.mentagrophytes* and *T.rubrum* as well as *M.canis* and *M.equinum*

#### Questions

1. How do you do hair perforation test?

Place a filter paper strip into bottom of a sterile petri dish and cover surface of strip with sterile distilled water. Add small portion of sterilized pre pubertal hair (contains no fatty acids that prevent growth of the fungus) into water. Add 5-6 drops of 10% yeast extract to speed up the reaction. Inoculate colony directly on hair and incubate at 25 deg C for four weeks.

2. When do you do hair perforation test?

This test is done to differentiate between dermatophytes *T.mentagrophytes* and *T.rubrum* as well as *M.canis* and *M.equinum*.

3. Why do you use prepubertal hair?

Pre pubertal hair is used as it does not contain fatty acid.

4. How do you interpret the test?

The test is positive for *T.mentagrophytes* and *M.canis* and negative in *T.rubrum* and *M.equinum*. It can be observed by conical perforation of hair shaft which is produced by *T.mentagrophytes* and not by *T.rubrum*.

#### 4.10. Macroscopic appearance of fungal cultures-exam point of view

Some of the macroscopic findings of fungal culture commonly kept in exams.

##### ***Penicillium spp.***

Obverse: Flat with velvety to powdery, greenish

Microscopy: Hyaline septate hyphae and the vesicles are absent. The conidiophore directly divides into elongated metulae from which flask shaped phialides arise which bear chains of conidia. Such an arrangement is called brush border appearance.

##### ***Aspergillus niger***

Obverse: The colonies are woolly dark brown to black.

Reverse: White to yellow.

Microscopy: Vesicle is globular shaped. The conidiophore is of variable length, phialides are biseriate i.e., arranged in two rows, covering entire vesicle, form radiate head. Conidia are black.

### ***Aspergillus fumigatus***

Obverse: The colonies are velvety or powdery, smoky green

Reverse: White to tan

Microscopy: Vesicle is conical shaped. Conidiophore is smooth. Phialides are single (uniseriate). Conidia arise from upper third of vesicle. Conidia are hyaline.

### ***Aspergillus flavus***

Obverse: Colonies are velvety, yellow to green or brown

Reverse: Golden yellow to red brown

Microscopy: Vesicle is globular shaped. Phialides are single and double cover entire vesicle. Conidia arise from entire vesicle. Conidia are hyaline.

### ***Mucor***

Obverse: Fibrous or cotton candy growth which is very vigorous called lid lifters.

Microscopy: The hyphae are broad with non-septate hyphae, with wide angle branching.

Sporangiophores are branched bearing spherical sporangia at tip of branch. The hyphae are without rhizoids or stolons.

### ***Rhizopus***

Obverse: Cottony growth with salt and pepper appearance due to sporulation

Microscopy: The hyphae are broad and non-septate with wide angle branching hyphae. They have thin walls submerged or aerial stolon with brown root like short branches i.e. rhizoids (nodal rhizoids).

### ***Candida spp.***

Macroscopy: White creamy, white with yeast odour.

Microscopy: Gram stain shows gram positive budding yeast cells.

*Cryptococcus spp.*

Macroscopy: Creamish-white yeast-like

Microscopy: Gram stain shows gram positive round yeast cells.

Negative stain: using Nigrosin to demonstrate the capsule.

Questions?

1. Give some examples of lab contaminants of fungus.

The common lab contaminants are *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Rhizopus* and *Mucor*.

2. How do you differentiate different spp of *Aspergillus*?

*Aspergillus niger*. Obverse -- colonies are dark brown to black

Microscopy---Conidiophores are of varying length, phialides are biserial (arranged in two rows) covering entire vesicle.

*Aspergillus fumigatus*--Obverse --Velvety or powdery to smoky green

Microscopy-- Conidiophores are smooth, phialides single (uniseriate) covers upper half of vesicle.

*Aspergillus flavus*--Obverse--Velvety, yellow to green or brown

Microscopy--Conidiophores are of varying length, phialides are single(uniseriate) and double cover entire vesicle.

3. How do you differentiate *Mucor* from *Rhizopus*?

*Mucor* is characterized by rapidly growing light dull to grey colonies and absence of rhizoids and no distinct columella. *Rhizopus* are characterized by nodal rhizoids beneath unbranched sporangiophores and ovoid columella.

4. How do you differentiate *Rhizopus* and *Aspergillus spp.* by microscopy?

*Rhizopus* is characterized by non-septate hyphae with wide angle branching whereas *Aspergillus spp.* are characterized by septate hyphae with acute angle branching of fungal filaments.

5. How do you differentiate *Candida spp.* from *Cryptococcus spp.*?

Both *Candida spp.* and *Cryptococcus* appear like Gram positive budding yeast cells in gram stain. They both can be differentiated by negative stain. *Cryptococcus* is a capsulated yeast which can be demonstrated by negative staining. *Cryptococcus* is urease positive whereas candida is urease negative.

6. Describe the morphological appearance of different dermatophytes that are observed in the lab.

	Obverse/reverse	Microscopy
<i>T. rubrum</i>	Obverse -Velvety. Reverse—red pigment	Microconidia-Teardrop, plenty. Macroconidia-- few long, pencil-shaped
<i>T. mentagrophytes</i>	Obverse-White to tan, cottony or powdery. Reverse-- Pigment variable	Microconidia—numerous round to pyriform. Macroconidia--cigar shaped, spiral hyphae
<i>T. violaceum</i>	Obverse- Very slow growing, waxy, Reverse--Violet to purple pigment	Macroconidia and microconidia –rare or absent. Distorted hyphae, Chlamydo spores seen.
<i>M. audouinii</i>	Obverse- Velvety, brownish, slow growing Reverse—no color change	Thick walled chlamydo spore, conidia rare, and irregular Microconidia and macroconidia-rare
<i>M. canis</i>	Obverse- Cottony, Reverse- orange pigment	Microconidia-- rare Macroconidia--Abundant, thick-walled spindle-shaped with up to 15 septa.
<i>M. gypseum</i>	Obverse -Powdery, cinnamon buff to brown colored Reverse- no color change	Microconidia--rare Macroconidia--Abundant thin walled with 4-6 septa.
<i>E. floccosum</i>	Obverse—powdery, greenish brown (khaki colored), raised and folded in centre. Reverse- no color change	Microconidia--absent Macroconidia--Club shaped

Note: *Trichophyton mentagrophytes* characterized by abundant grape like clusters of sub spherical microconidia.

*Trichophyton rubrum* -- characterized by tear drop shaped microconidia.

*Trichophyton mentagrophytes* --Urease is hydrolysed

*Trichophyton rubrum*-- Urease is not hydrolysed.

7. How much time is required for fungi to grow on culture media?

3 weeks is required for slow growing fungi like dermatophytes whereas contaminants require only 48 hours.

8. What are the different spp of *Candida*?

*Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Candida dubliensis*, *Candida guilliermondii*, *Candida glabrata*, *Candida kefyr*.

9. What are the diseases caused by *Candida albicans*?

Cutaneous --Intertrigo, Paronychia, onychomycosis, Diaper dermatitis, *Candida granuloma*

Mucocutaneous- Oral thrush, glossitis, chelitis, vulvovaginitis, balanitis, chronic mucocutaneous candidiasis, systemic endocarditis, meningitis, candidemia, arthritis, pulmonary candidiasis

10. What is corn meal agar?

Corn meal agar is a nutritionally deficient medium used for demonstration of chlamydospore production by *Candida albicans* and *Candida dubliniensis*.

10. What is chlamydospore?

The chlamydospore has been defined as a thick-walled, non-deciduous, intercalary or terminal, asexual spore formed by the rounding of a cell or cells produced under nutrient poor media such as corn meal agar, Staib's agar, Pal's agar, Caesin agar and tobacco agar.

11. How do you identify *Cryptococcus spp*?

*Cryptococcus spp* is identified by detection of cryptococcal antigen by immunodiagnostic tests of the serum and the CSF provides a definitive diagnosis of the infection. Different techniques such as latex agglutination, enzyme-linked immunosorbent assay (ELISA), and lateral flow assays can be used to detect cryptococcal antigen.

12. What are the different diseases manifestation by *Cryptococcus neoformans*?

Pulmonary cryptococcus – pneumonitis

Osseous cryptococcosis-Presents with osteolytic lesions

Cutaneous cryptococcosis presents as primary and secondary manifestations

CNS cryptococcosis- meningitis

Visceral cryptococcosis- the common primary site of infection is lung.

13. Why pigeons don't get *Cryptococcus* infection even after having *Cryptococcus* fungus?

The body temperature of pigeons helps in preventing infection with *Cryptococcus*.

14. Name some dermatophytes.

Trichophyton, Microsporon and Epidermophyton.

15. Which part of the body is affected by dermatophytes?

Hair, skin and nail are affected by dermatophytes.

16. What type of fungi are dermatophytes?

Dermatophytes are molds (filamentous fungi)

17. How do you identify different spp. of Trichophyton?

Ans explained in the tabular column.

18. Which fungi give positive urease test?

*Trichophyton mentagrophytes* give positive urease test.

*Cryptococcus spp.* is urease positive.

19. What special culture media are used for growing dermatophytes?

Dermatophyte test agar is used for growing dermatophytes

20. How do you identify Microsporon?

Ans explained above in the tabular column.

21. What are the different spp of *Microsporon*?

*M. audouinii*, *M. canis*, *M. cookei* complex [Genus *Paraphyton*] *M. ferrugineum* *M. gallinae* [*Lophophyton gallinae*] *M. gypseum* complex [Genus *Nannizzia*] *M. nanum* [*Nannizzia nana*] *M. persicolor* [*Nannizzia persicolor*] *M. praecox* [*Nannizzia praecox*] *M. racemosum* *M. vanbreuseghemii* [*Lophophyton*]

22. What are zoophilic, geophilic and anthropilic fungi?

Anthrophilic	Zoophilic	Geophilic
<i>T. concentricum</i>	<i>T. equinum</i>	<i>M. gypseum</i>
<i>T. megnini</i>	<i>T. mentagrophytes</i>	<i>T. ajelloi</i>
<i>T. tonsurans</i>	<i>M. canis</i>	<i>M. fulvum</i>
<i>M. audouinii</i>	<i>M. persicolor</i>	<i>M. nanum</i>
<i>E. floccosum</i>	<i>M. equinum</i>	<i>E. stockdaleae</i>
<i>T. violaceum</i>		

23. What are ectothrix and endothrix?

Endothrix. Microscopic examination of infected hairs reveal the hair shaft filled with masses of large arthroconidia (4 to 7  $\mu\text{m}$ ) in chains.

Ectothrix. Microscopic examination of infected hair shows external masses of spores that ensheath the hair shaft.

24. Which clinical specimens are used for diagnosis of infections with dermatophytes?

Nail, Skin scrapings and hair are used.

25. Name different spp of Epidermophyton

*E.floccosum* and *E.stockdaleae*.

26. What are the diseases caused by *A.fumigatus*?

*A.fumigatus* accounts for acute pulmonary and allergic aspergillosis.

27. What are the diseases caused by *A.niger*?

*A.niger* can cause invasive infection but more commonly colonizes the respiratory tract and causes otitis externa.

28. What is aspergilloma?

An aspergilloma is a clump of mold which exists in a body cavity such as a paranasal sinus or an organ such as the lung. By definition, it is caused by fungi of the genus *Aspergillus*. It forms in preexisting cavities in lung. They are usually solitary vary in size approximately 8-10 cm in diameter.

29. Name some immunological test used for diagnosis of *Aspergillus spp*

ELISA test for detecting galactomannan for early diagnosis of *Aspergillus*. In addition,

BALISA (biotin – avidin amplifications system for antibody detection).

30. What is aflatoxin?

Aflatoxins are secondary metabolites produced by *Aspergillus flavus*, *A.parasiticus*, *A.nomius*

Source of exposure include nuts, maize and they present with Aflatoxicosis, Reye's syndrome, Hepatitis, Hepatoma.

31. What are dermateaceous fungi?

These are pigmented fungi darkly colored which may be found as yeast or hyphal form.

Eg. *Aureobasidium pullunas*, *Wangiella dermatitidis*, *Exophiala jeanselmei*, *Exophiala spinifera* and *Hortaean weneckii*.

32. What is mucormycosis?

Spores of fungi causing mucormycosis are found ubiquitously in the environment. Transmission occurs via inhalation, inoculation or rarely ingestion of spores. Spores develop into mycelial form containing wide aseptate hyphae which are angioinvasive and indicates spread of infection.

Predisposing factors: Agents of mucormycosis require iron as growth factor. Hence conditions with increased iron load are at higher risk of developing invasive mucormycosis.

Diabetic ketoacidosis (DKA) is the most important risk factor. Acidosis causes release of iron from the sequestered proteins in serum in end stage renal disease. Patients taking iron therapy or deferoxamine (iron chelator), defects in phagocytic functions (e.g., neutropenia or steroid therapy).

33. What is the morphology of *Penicillium marneffeii*?

LPCB mount of the colonies reveals hyaline thin septate hyphae, and the vesicles are absent. The conidiophore and its branches give rise to elongated metulae, from which flask-shaped phialides originate which bear chain of conidia giving a brush border appearance.

*Penicillium marneffeii* is a thermally dimorphic fungus that causes opportunistic infection in HIV-infected patients.

34. What are the diseases produced by *Fusarium spp*, *Alternaria spp* and *Curuvularia spp*?

*Curvularia spp.* causes keratomycosis, sinusitis, onychomycosis, phaeohypomycosis, eumycetoma

*Fusarium spp* causes fungal keratitis.

*Alternaria spp.* causes keratomycosis,

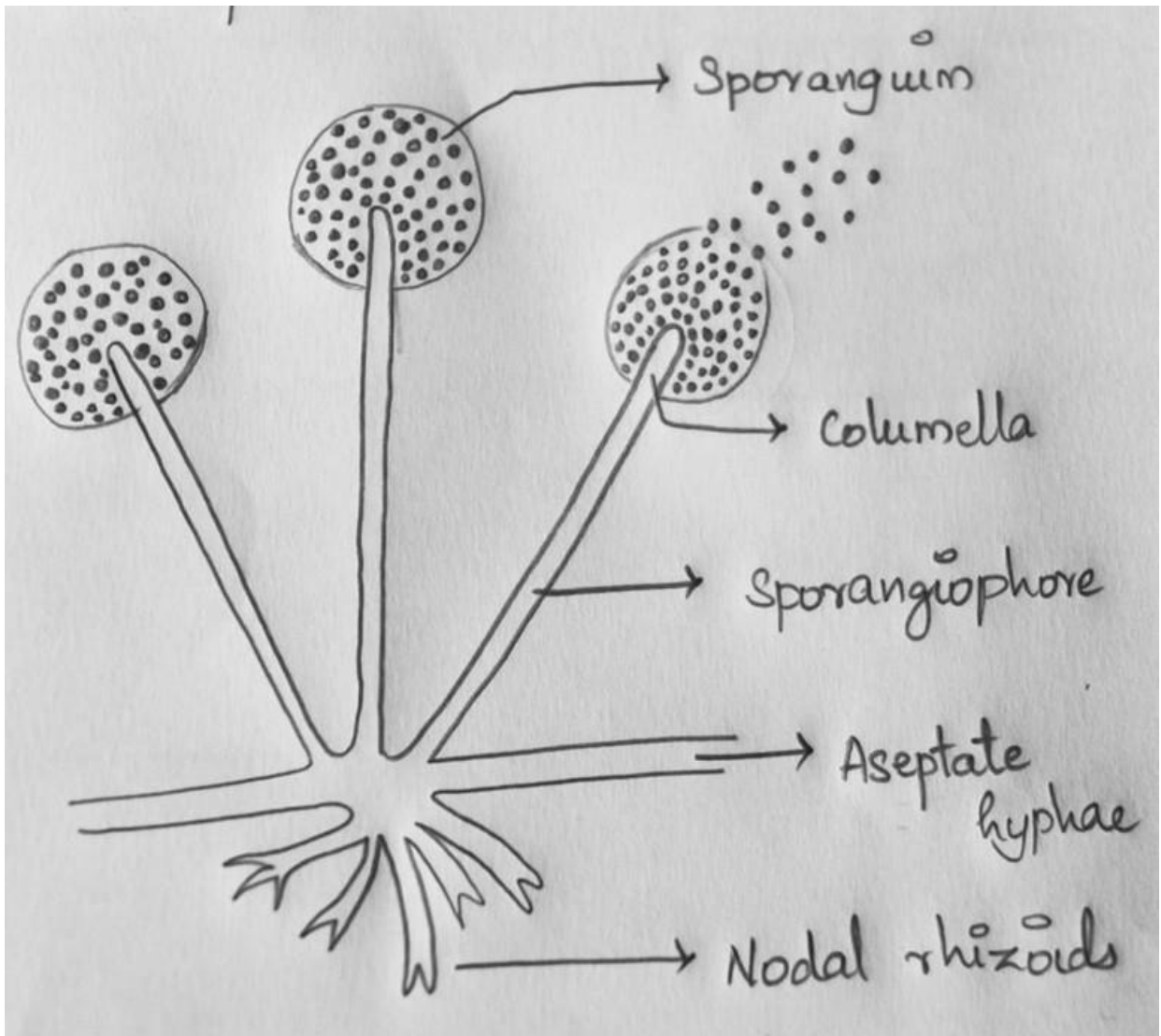
35. Why acidic pH is kept in SDA?

The pH is adjusted to acidic pH to enhance the growth of fungi and to inhibit the bacterial growth.

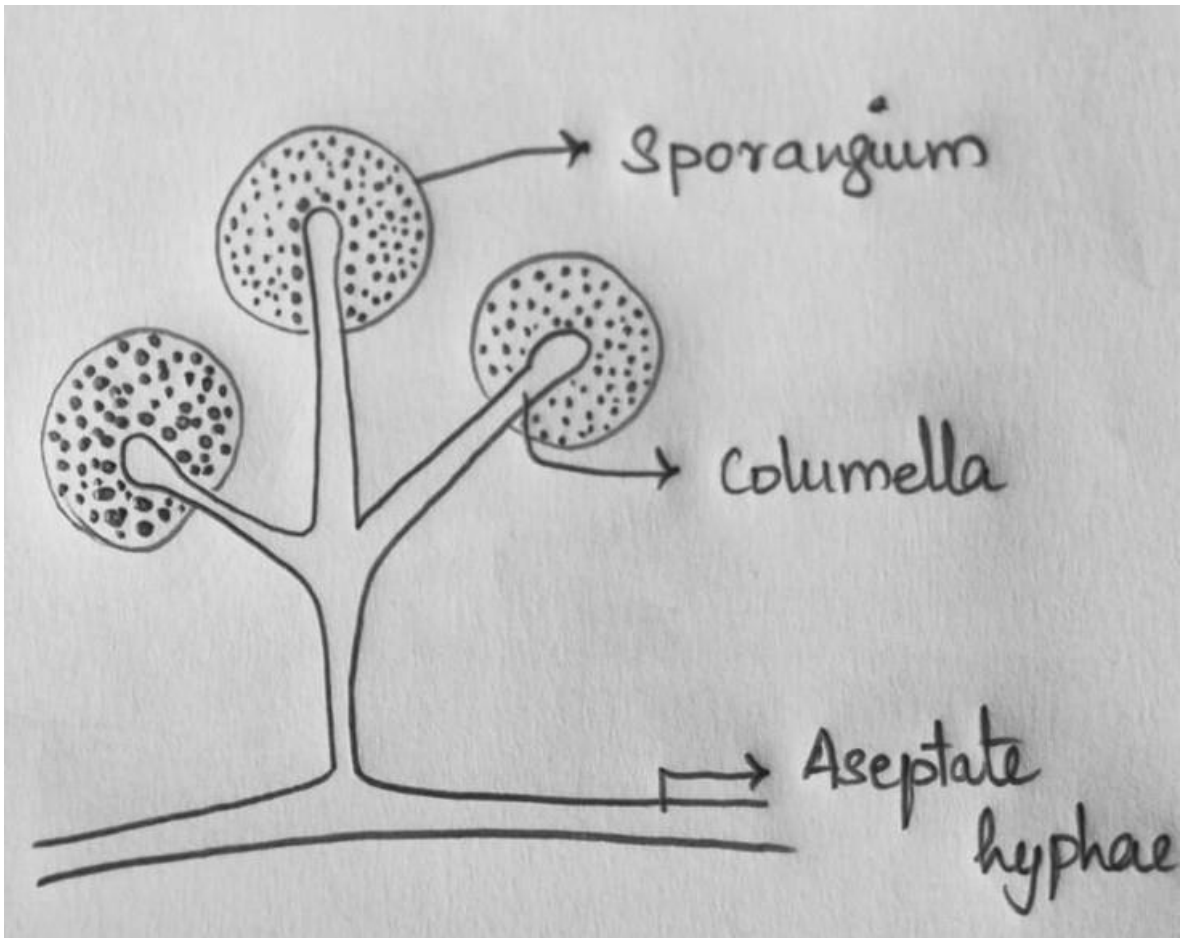
36. What is calcofluor white stain?

Calcofluor white stain is a fluorescent stain specifically for the identification of onychomycosis fungal infection especially of *Candida spp.* The chitin containing structures fluoresce bright white under ultraviolet light in a fluorescent microscope.

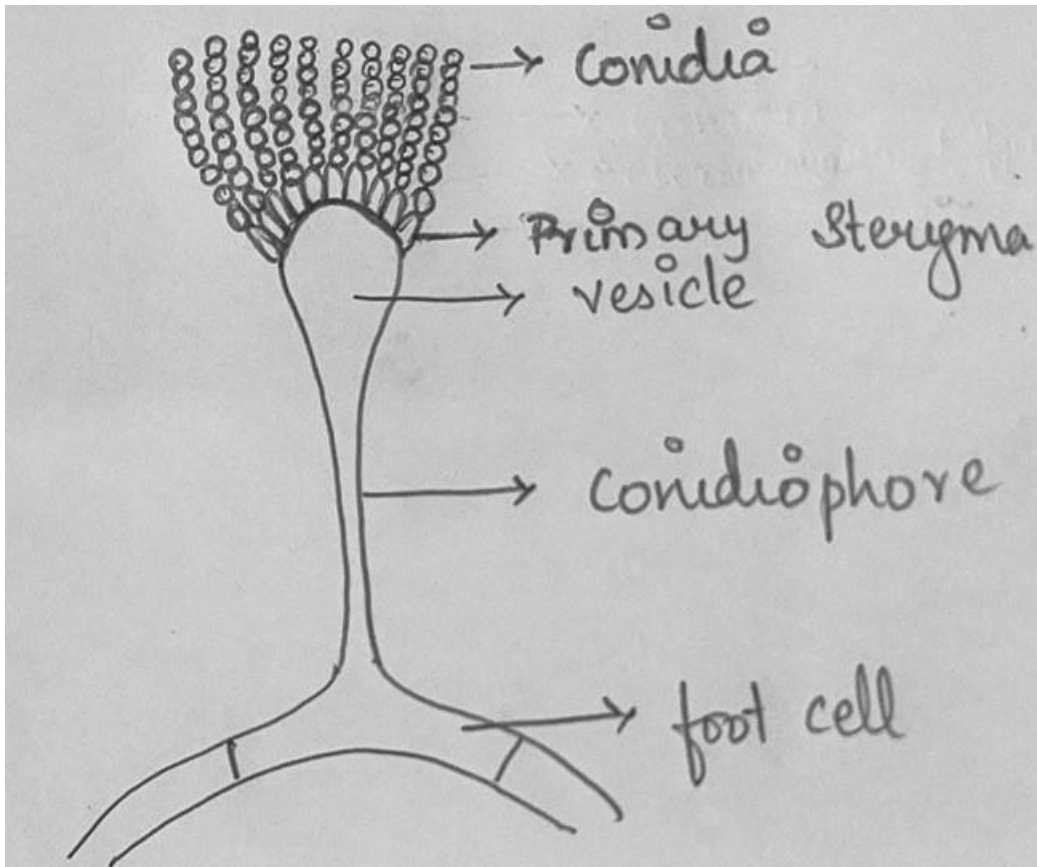
### MICROSCOPIC APPEARANCE OF VARIOUS FUNGUS



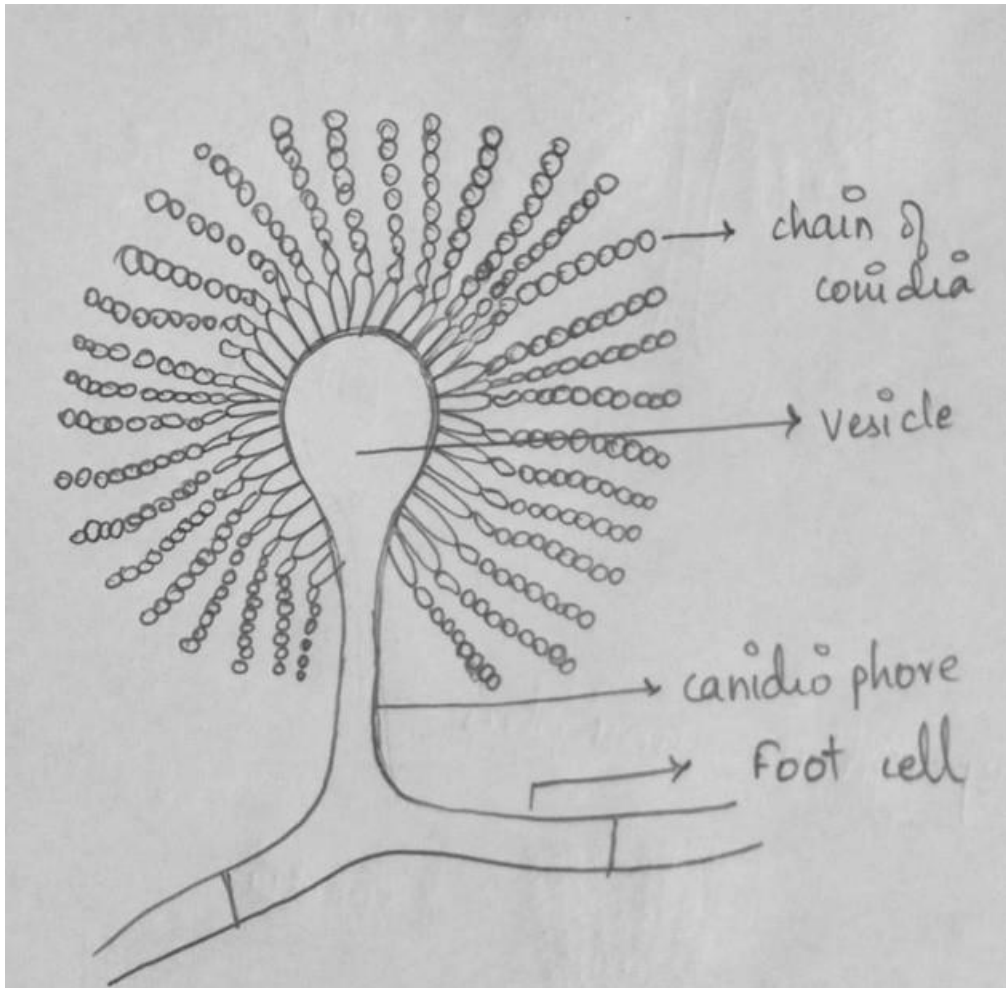
**RHIZOPUS**



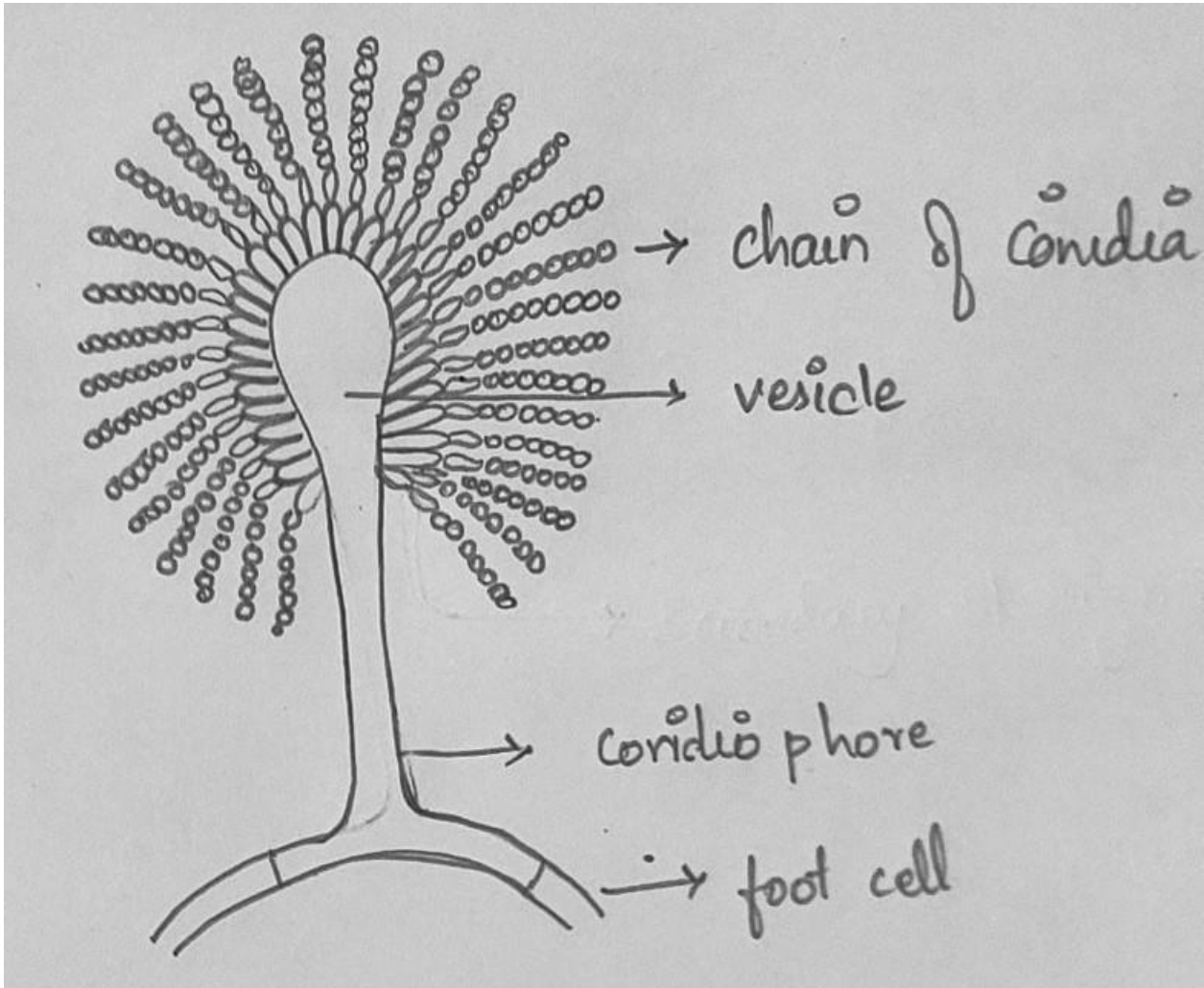
**MUCOR**



**ASPERGILLUS FUMIGATUS**

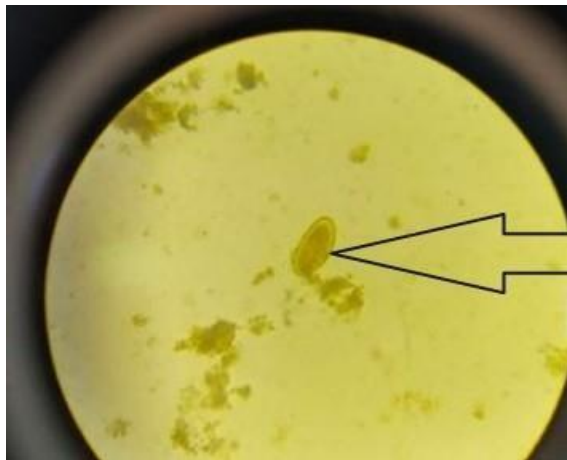


**ASPERGILLUS NIGER**



**ASPERGILLUS FLAVUS**

## 5. PARASITOLOGY



## 5.Parasitology

### 5.1. What should you know?

Here you should know about stool examination both macroscopic and microscopic and should know various concentration methods of stool examination.

As a student you may not like to see the stool, but this is important as it carries 20 marks. In the practical exam you will be asked to focus saline and iodine mount of stool sample.

Reasons for examining stool sample for parasites.

1.To identify the parasitic causes of blood and mucus in faeces and differentiate amoebic from bacillary dysentery.

2.To identify intestinal parasitic infection that requires treatment. That is those associated with weight loss, persistent diarrhea, intestinal malabsorption.

**5.2. Collection of sample:** The sample should be collected fresh in a wide mouth container. The sample should not be mixed with urine. It should not be collected in narrow neck bottles, match box etc. Samples from diapers in children are not acceptable. In case of bedridden patients with diarrhea, rectal swabs are accepted. A large teaspoon amount of faeces is adequate or about 10ml of a fluid specimen is adequate. We may need to collect several specimens on alternate day when the parasites are excreted intermittently as in *Giardia*.

Sample should reach lab within 1 hour of being collected. Dysentric and watery specimens must reach the lab as soon as possible preferably within 15 min as motile parasites such as *E.histolytica* and *G.lambliia* may not be detected if there is delay.

**Caution.** Faeces specimens should be handled with care as apart from parasites some bacteria like *V.cholerae*, *Shigella spp.* *Salmonella spp* and viruses like HIV, Hepatitis viruses, and rota virus can cause infection.

**Note:** Ask for history of spinach or beetroot intake before they give the sample which can cause change in colour of stool.

Macroscopic examination of stool sample is necessary before you proceed with microscopic examination.

### 5.3. Macroscopic examination of stool.

Look for colour of the specimen.

Consistency: formed, semi formed, watery, unformed.

Presence of blood, pus, mucus mixed with faeces.

Specimen contains worms or segment of worms(*Taenia*)

## Macroscopic appearance of faecal specimens in some diseases

Appearance	Possible cause
Unformed containing pus, mucus mixed with blood	Shigellosis Campylobacter enteritis EIEC dysentery
Unformed pus mixed with blood (acidic pH)	Amoebic dysentery
Watery stools	EPEC, ETEC diarrhea, Rota virus enteritis, Cryptosporidiosis
Rice water stools	Vibrio cholerae
Unformed, pale colored frothy, unpleasant smelling stools that float on water (high fat content)	Giardiasis, other conditions that cause malabsorption e.g., post infective malabsorption
Unformed or semi formed black stools	Malena (gastrointestinal bleeding), hook worm disease, iron therapy.

### 5.4. Microscopic examination of stools.

Saline and Iodine amount

The normal stool constitutes muscle fibres, glycogen, air cells, fat globules and hair. The wet mounts commonly used for stool detection are

Saline mount

Iodine mount

The wet mount preparations of stool specimen are screened first under low power objective and then under high power objective. The saline mount is done to identify the motility of the parasite and iodine mount is done to identify the cyst. The iodine mount is used to stain glycogen and nuclei of cyst if present.

Procedure for both saline and iodine mount

Take a clean glass slide.

With the help of a glass dropper, put a drop of saline on one end of the slide.

Put a drop of iodine on the other end of slide.

With the help of an applicator stick apply a small portion of stool sample and emulsify

Put a cover slip over both the saline and iodine suspension of the stool.

Examine the preparation first under low power and then under high power.

Record the findings with description of morphological feature.

**Note**—Stool wet mount should be screened immediately.

The examiner commonly checks whether you have adjusted the microscope properly for 10X and 40X and asks questions about use of saline and iodine mount.

The iodine commonly used are Lugol's iodine which contains iodine and potassium iodide in distilled water. Other staining methods that can be done for stool are Gram stain, from the culture of stool, acid fast stain (1%) and Trichrome stain. In acid fast stain, we use 1% H<sub>2</sub>SO<sub>4</sub> as decolorizing agent.

The procedure to make preparation of stool smear is like any other smear. The principle is that oocyst of coccidian parasites are acid fast and retain the basic dye (dilute carbol fuchsin) appearing pink.

Oocysts of *Cryptosporidium* and *Isospora* are consistently acid fast in nature while *Cyclospora* is variably acid fast. *Cyclospora* oocysts are twice the size of the *Cryptosporidium* oocysts. *Cryptosporidium* oocysts are to be distinguished from yeast cells, which are non-acid fast and show budding and variable sizes. If the consistency of stool is watery and looks like rice water stool, you can see the motility of bacteria by hanging drop method. Gram stain can be done if you suspect *Vibrio cholerae* which shows comma shaped bacteria. Remember if you suspect *Vibrio* you need to inform the physician immediately as the treatment should be started immediately and also inform the municipal authority as it is a notifiable disease.

If the egg concentration is low then we need to do concentration techniques. Concentration method is not done routinely.

## 5.5. Concentration methods of stool

Concentration of stool is recommended when direct examination of wet mount of stool fails to demonstrate any parasites, particularly when the number of parasites in stool specimens are low. Remember trophozoites cannot be concentrated as they get killed.

Concentration techniques may be required:

To detect *Strongyloides* larvae, the eggs of *Taenia*, cysts of *G.lambliia*.

To detect small parasites, e.g. small fluke eggs. Oocysts of intestinal coccidia prior to staining.

To quantify intestinal parasites.

Different types of concentration methods are

Saturated salt solution method

Formalin ether concentration method

Zinc sulphate floatation methods

Sheather's sucrose floatation method

### **5.5.1. Saturated salt floatation method**

In saturated salt solution floatation method, saturated salt solution, liquid with high specific gravity is used. In this method the eggs and cysts which have less specific gravity will float. Saturated salt solution is prepared by dissolving a spoonful of sodium chloride in 100ml of distilled water.

#### **Procedure**

1. Take a walnut sized stool sample (approximately 1gram) in a 30ml glass vial.
2. Add a few ml of saturated sodium chloride solution to the stool and mix it with a broom stick.
3. When the stool suspension is smooth, add a few more ml of salt solution and mix.
4. Keep repeating steps 2 and 3 until the container is completely full with continuous stirring.
5. After all the salt has dissolved completely without a trace, more salt is added till it remains undissolved. This makes saturated salt solution.
6. Place a broad cover slip over the glass vial, so that it is in contact with the top of the meniscus.

#### **Advantages**

- Simple procedure
- Easy to perform
- Least subjective to technical error.
- Good method for concentration of parasitic eggs.

#### **Disadvantages**

Not suitable for demonstration of unfertilized eggs of *A. searisi*, operculated eggs of trematodes, larvae of *Strongyloides* and eggs of *Taenia* species.

High specific gravity of the fluid may cause distortion of morphology of the parasitic eggs and cysts.

### 5.5.2. Formalin ether sedimentation method

#### Procedure

Take a half teaspoon of stool in 15ml centrifuge tube containing 10ml of 10% formalin and allow it to stand for 30 minutes.

Filter the faecal suspension through two layers of gauze placed in a funnel into a 15ml centrifuge tube. Add saline to the tube to bring the fluid level within a few millimeters of the rim of the tube.

Centrifuge the tube for 10 min.

Discard the supernatant. Suspend the sediment in saline, nearly filling up to the brim of the tube.

Centrifuge the tube again for 10min at 500xg.

Resuspend the sediment in 7ml of 10% formalin, and 3ml ether.

Close the tube with a stopper and shake it well for 30 seconds.

8. Remove the stopper carefully

9. Centrifuge the tube at 500xg for 10 minutes. Allow the tube to stand 5 min.

After the centrifugation 4 layers are formed namely

First sediment layer at the bottom of the tube containing parasitic cysts or

Second layer of formal saline,

Third layer of faecal debris on the top of the formal saline layer and

The top layer of ether.

1. Remove the plug of faecal debris by piercing all around with an applicator stick, taking care not to disrupt the debris.

2. Discard all the fluid into the discarding jar by one firm swing, leaving behind one or two drops of fluid with the sediment.

3. Mix the sediment with the fluid using an applicator stick. Then with a pasteur pipette, aspirate a little of the sediment fluid and examine by making the saline and iodine wet mount preparations.

Examine under 10X and under 40X objective.

## Advantages

More sensitive method

Morphology of eggs and cysts preserved.

Good method for both cysts and ova.

## Disadvantages

Technically cumbersome.

Ether is highly inflammable and not easily available.

**Note:** These two are common concentration methods done.

**5.5.3 & 5.5.4. Other concentration methods.** In addition, Sheather's sucrose floatation method is done for coccidian parasites. Zinc sulphate floatation technique (where we use 33% zinc sulphate solution) is recommended for concentrating the cysts of *G.lambli*a, *E.histolytica* ~~/*E.dispar*~~, and the eggs of *T.trichuria* and *Clonorchis spp*. The procedure is the same as that of saturated salt solution.

An increasing number of parasites are being associated with human immunodeficiency virus (HIV) infection and AIDS. *Cryptosporidium*, *Isospora belli*, *Cyclospora* are commonly observed in addition to *Strongyloides stercoralis*

## 5.6. Morphological forms seen in stools

Faces	<i>E. histolytica</i>	Cyst, trophozoite
	<i>G. lamblia</i>	Cyst, trophozoite
	<i>T. hominis</i>	Trophozoite
	<i>B. coli</i>	Cyst, trophozoite
	<i>I. belli</i>	Oocyst
	<i>C. parvum</i>	Oocyst
	<i>C. cayetanensis</i>	Oocyst
	<i>Microsporidia</i>	Spores
	<i>T. solium</i>	Egg, segment
	<i>T. saginata</i>	Egg, segment
	<i>H. nana</i>	Egg
	<i>D. latum</i>	Egg
	<i>C. sinensis</i>	Egg
	<i>F. hepatica</i>	Egg
	<i>F. gigantica</i>	Egg
	<i>Schistosoma spp.</i>	Egg
	<i>A. lumbricoides</i>	Egg, worm
	<i>E. vermicularis</i>	Worm (sometimes eggs are found in faces)
	<i>S. stercoralis</i>	Larva
	<i>T. trichura</i>	Egg
	<i>A. duodenale</i>	Egg
	<i>N. americanus</i>	Egg
	<i>Paragonimus spp</i>	Egg (eggs are more commonly found in sputum)

## 5.7. Hanging drop experiment

### Requirements:

Cavity slides measuring 75mm length X 25mm width X 1.25 mm thickness

Coverslip No 1.

Bunsen burner / spirit lamp

Diamond pencil or lead pencil

Bright field microscope with objectives of 10X , 40X and 100X and eyepieces of 10X

Paraffin wax / mineral oil

1% sodium hypochlorite is used as disinfectant solution for decontaminating the viewed slides.

The viewed slides are put into a jar containing 1% sodium hypochlorite for disinfection.

### Procedure

Place a cover slip on the table and in each corner of the coverslip place a drop of paraffin wax / mineral oil.

In the centre of the coverslip, place a loopful of the watery portion of the faeces or of the broth culture. Insert a cavity slide over the coverslip and press gently.

Turn the slide so that the coverslip is on top and inspect whether the drop is hanging freely. Examine under the microscope, focusing first on the edge of the drop at low magnification and then switch to the higher objective (40X)

### Quality control:

*E.coli* ATCC 25922 should appear motile

*Klebsiella pneumoniae* ATCC will appear non motile

**Interferences:** Brownian movement may give a false appearance of motility.

**Critical alerts:** Detection of darting motility resembling *Vibrio cholerae* is immediately reported. Although stool culture is reported in bacteriology, it has been covered to a extent in parasitology so that it will be complete.

## 5.8. Stool culture

**Background:** Enteric pathogens include *Salmonella* and *Shigella spp.*, *Campylobacter*, *E. coli* O157:H7, *Yersinia enterocolitica*, *Clostridium difficile*, *Aeromonas* and *Plesiomonas*. *Vibrio cholerae* and *V. parahemolyticus* are an important cause of diarrhea in other parts of the world. *S. aureus* and *Candida albicans* may be found in pure cultures from patients on broad-spectrum antibiotic therapy.

**Principle:** Detect bacterial pathogenic organisms in the stool; Enteric fever A,B, bacillary dysentery, non-typhoidal *Salmonella* infection. Stool sample should be collected during the symptomatic phase of illness for optimal recovery of pathogens. Verification and validation of media, biochemical and antibiotic discs are done regularly as part of QC. Common pathogens and commensals are explained in chart below

Types of specimen: Stool or rectal swab or stool (fresh random) in fecal transport system.

**Patient preparation:** Instruct the patient on how the specimen should be collected and transferred to the container. Provide him/her with sticks and containers. Submit one specimen per stool sample, to a maximum of 2 stool samples, ideally on separate days.

Common pathogens	Commensal flora
<i>Helicobacter pylori</i>	<i>Enterobacteriaceae</i> other than the common pathogens
<i>Salmonella spp</i>	<i>Bacteroides spp.</i>
<i>E. coli</i> O157:H7	<i>Streptococcus spp.</i>
<i>Campylobacter spp.</i>	<i>Pseudomonas spp.</i>
<i>Staphylococcus aureus</i>	<i>Lactobacilli</i>
<i>Vibrio cholerae</i>	<i>Coagulase negative Staphylococci</i>
<i>Yersinia enterocolitica</i>	<i>Bacteroides</i>
<i>Shigella spp</i>	<i>Peptostreptococcus</i>

**Specimen collection:** The specimen should contain at least 5 g of faeces and if present, those parts that contain blood and or mucus should be selected. The specimen should not be contaminated with urine. Close the lid.

Rectal swab: Rectal swabs may be adequate for the detection of pathogens in acute infections but are NOT the preferred specimens. Pass swab beyond anal sphincter, carefully rotate, and withdraw.

Swabbing of lesions of rectal wall or sigmoid colon during proctoscopy or sigmoidoscopy is preferred.

Who will collect the specimen?

The patient. If stool is unobtainable, nursing staff or physician will collect fecal swab.

Volume: at least 5 g of faeces.

Time lapsed before processing the sample: Stool samples should be examined and cultured as soon as possible.

after collection. As the stool specimen cools, the drop in pH will inhibit the growth of most *Shigella spp.* and some *Salmonella spp.* Freshly passed stool specimens should be transported to the laboratory within 30 minutes of collection and processed in 2 hours. If there is a delay in transport and/or processing, the specimen should be transferred into a transport container with Cary-Blair medium. The media used are

Xylose Lysine Deoxycholate (XLD)

Selenite F broth

When requested or clinically indicated culture to:

Blood Agar (BA)

Thiosulfate Citrate – Bile Salts – Sucrose (TCBS) - selective for *Vibrio*

For biochemical reactions if growth occurs. (refer biochemical reactions which is explained in bacteriology)

## **Procedure**

Direct Examination:

**Macroscopic appearance:** Colour, consistency, presence of blood/mucus and parasitic segments is noted. Perform hanging drop in case of rice watery stools/ clinical request/ suspicion of cholera.

Culture:

1 gram of stool is transferred to a tube of Selenite-F broth and is incubated for 6 to 7 hours in the broth. Then it is subcultured on Mac conkey agar, XLD and DCA agar. The plates are incubated at 37deg C. Suspicious colonies of enteric pathogens should be sub cultured and identified using the laboratory identification system. i.e. conventional or automated methods as applicable.

Antimicrobial sensitivity testing:

Identify isolates and perform susceptibility testing as per laboratory protocol. **The drugs commonly used are Ampicillin, Nalidixic acid, Furazolidone, Ciprofloxacin.**

**Potential source of variations:** Heavy growth of the normal commensal flora can mask the identification of the pathogens which are present in small numbers. Also, delay in processing and inappropriate selection of the portions of the sample can hamper the culture results.

**Interferences:** If the patient is already on antibiotics, the culture can come as negative. Samples with No enteric pathogens– will be reported after 48 hours of incubation.

Turnaround time: Isolation of a possible pathogen can be expected after 2-3 days. Negative culture will be reported 1-2 days after the receipt of the specimen.

**Critical values:** Detection of *Vibrio cholerae* in culture/ hanging drop positive are phoned out to the clinician.

Lab clinical interpretation:

A positive report will be issued only in case of isolation of *Salmonella*, *Shigella* and *Vibrio*, otherwise report will be issued as “No enteric pathogens”. or No *Salmonella*, *Shigella* grown in culture.

### Questions

1. List the normal constituents of stool.

The normal constituents are yeast cells, cotton fiber, starch cell, potato parenchymal cell, plant epidermal hairs, fungal spores, muscle fibres, vascular structure of plants, pollen, stone cell, plant cells, oil drops, rice, starch, potato starch, corn starch, leucocytes, moulds and bacteria.

2. What are the different wet mount preparations of stool?

Different wet mount preparation of stool are saline mount, iodine mount, LPCB wet mount and buffered methylene blue.

3. What are the uses of saline mount?

The advantage of saline mount is to identify the motility and also to see trophozoite and larva clearly.

4. What are the use of iodine mount?

### Advantage.

a. The number of nuclei present in the protozoan cyst can be clearly distinguished

b. It also demonstrates the yellowish cytoplasm and brown glycogen mass present in the cyst.

### Disadvantage

The bile staining property of the helminthic eggs cannot be made out.

Trophozoites are killed by iodine, hence cannot be demonstrated in iodine wet mount.

5. List some bile-stained eggs.

Eggs of *Ascaris lumbricoides*, *Trichuris trichura*, *Taenia* spp. *Echinococcus* spp., *Diphyllobothrium latum*, *Schistosoma mansoni*, *Schistosoma japonicum*, *Fasciola hepatica*, *Fasciolopsis buski*, *Paragonimus westermani*, *Clonorchis sinensis*.

6. List some non-bile-stained eggs.

Eggs of Hook worm (*Ankylostoma duodenale*), *Enterobius vermicularis* and *Hymenolepis nana*.

7. List the intestinal and non-intestinal coccidian parasites.

Intestinal coccidian parasites

*Cryptosporidium parvum*, *Cyclospora cayetanensis*, *Isospora belli*

Non- intestinal coccidian parasites

*Toxoplasma gondii* *Sarcocystis hominis*.

8. How do you prepare wet preparation?

The wet preparation of stool sample is done by keeping the stool sample on a slide. A coverslip is put and examined under 10X and then under 40x. Look for the motility of the parasite.

9. What is the importance of looking for pus cells and RBCs?

Pus in the stool is an indication of infection. Mucus in the stool can be related to infection, inflammation, cancer, constipation, or conditions of the anus or rectum. The frequent, loosely formed stool can be related to diet, medications, infection, inflammation, or food poisoning.

10. Which is the heaviest ova?

Unfertilised eggs of *Ascaris lumbricoides*, Eggs of all intestinal flukes, eggs of *Taenia solium* and *Taenia saginata*.

11. Give some examples of parasites found in urine.

*Wucheria bancrofti*, *Schistosoma hematobium*, *Trichomonas vaginalis*.

12. Give some examples of viviparous nematodes.

*Wucheria bancrofti*, *Trichinella spiralis*, *Dracunculus medietensis*, *Brugia malayi*, *Brugia timori*.

13. Which parasitic ova can float in saturated salt solution?

Eggs of *Ankylostoma duodenale*, *Enterobius vermicularis*, *Necator americanus*, *Trichuris trichura*, *Ascaris lumbricoides*.

14. How are oocysts of *Cryptosporidium* differentiated from yeast cells in this staining technique?

The oocysts of *Cryptosporidium* are acid-fast while that of the yeast cells are non- acid fast and are of variable sizes with budding.

15. Name the coccidian parasite associated with traveller's diarrhoea.

*Cyclospora cayetanensis* and *Cryptosporidium parvum*.

16. Mention the different ways of staining methods for detection of cyst/eggs in stool.

Saline mount, iodine mount, Gram stain, Trichrome staining method, Modified acid fast stain.

17. What are the different concentration methods used for detection of cysts/eggs in stools?

Saturated saline floatation method, Formalin ether sedimentation method, Zinc sulphate floatation method, Sheather's sucrose floatation method, Magnesium sulphate floatation method.

18. Which is the stool concentration method employed for recovery of coccidian parasite?

The stool concentration method employed for the recovery of coccidian parasites is the Sheather's sucrose floatation method.

19. List all acid-fast parasitic components.

1. *Cryptosporidium parvum*

2. *Cyclospora cayetanensis*

3. *Isospora belli*

4. *Cyclospora*

20. What are the advantages of floatation method?

21. What are the disadvantages of floatation method?

22. What are the advantages of sedimentation method?

23. What are the disadvantages of sedimentation method?

Answers for questions 20, 21, 22 and 23 are given in the explanation above.

24. List the selective medias used for isolation of stool sample.

XLD and DCA are selective medias used for isolation of pathogenic organism in stool. TCBS is used as aselective media for isolation of *Vibrio spp* from stools.

25. Which is the transport media used if there is delay in transporting stool sample to lab?

Cary blair transport medium and Alkaline peptone water for *Vibrio cholerae* are the transport medium used.

26. What is Selenite F broth and its use?

Selenite F broth is an enrichment medium. This media is used when pathogens like *Salmonella spp.* and *Shigella spp.* to be recovered from stools.

27. How do you process the sample from selenite F broth?

The stool sample should be inoculated in selenite F broth for 6 to 7 hours. This time period is required as selenite inhibits the growth of *Escherichiae coli* and allows *Salmonella spp.* or *Shigella spp.* to grow. After 6 to 7 hours the sample should be subcultured on Mac conkey, XLD and DCA agar.

**Note:** Prolonged inhibition in selenite F broth further inhibits *Salmonella spp.* or *Shigella spp.* and allows *Escherichiae coli* to grow. So the timings are important.

28. How do you confirm the organism after identifying with biochemical reactions?

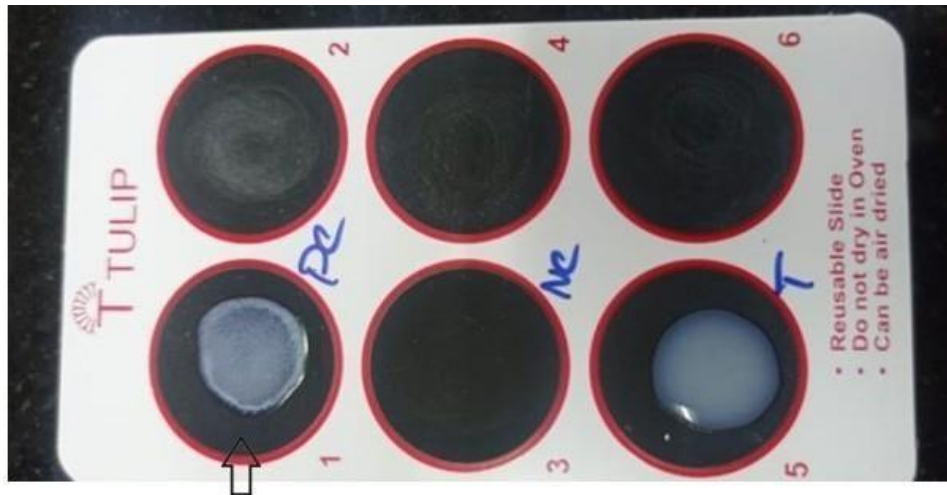
In case of *Salmonella*, *Shigella* and *Vibrio spp.* after the organism is identified by biochemical test, antisera are used to confirm the identification. Slide agglutination is done with the organism and type specific antisera.

In case of *Salmonella* the identification is based on Kauff mann white scheme classification.

This classification is based on presence of O and H antigen which can be detected by agglutination with specific antisera.

For eg *Salmonella typhi* contains group specific antigen type 9 (serogroup D) a loop full of typhoid O antiserum is added to one drop of bacterial emulsion on the slide and agglutination is looked for after rocking the slide gently; prompt agglutination indicates Group D *Salmonella*. Similarly agglutination with flagellar antiserum (anti -d serum ) confirms *S Typhi* .

## 6.SEROLOGY



## 6.1 Rapid plasma reagin test

The rapid plasma reagin (RPR) is a macroscopic, nontreponemal flocculation card test used to screen for syphilis. It is a non-specific test. The RPR test is used to detect and quantify reagin IgM and IgG antibody present in serum or plasma from persons with syphilis, or with other treponemal diseases.

### **Principle:**

The test antigen consists of cardiolipin-lecithin-coated cholesterol. 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. If antilipoidal 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 grey.

### Qualitative

1. Using a fresh disposable serum dropper for each sample, hold the dropper in a vertical position, dispense one free-falling drop (0.05 ml) of serum or plasma sample onto a circle on the test card.
2. Using the flat end of the serum dropper spread the sample over the entire area of the test circle.
3. Squeeze the dropper bottle and draw a sufficient volume of the antigen suspension into the bottle. Dispense several drops into the dropper bottle cap to make sure the dropper passage is clear. Prior to dispensing carbon antigen, agitate the dropping bottle for a few seconds to ensure reagent homogeneity.
4. Dispense one free-falling drop of the antigen suspension onto each sample while holding the dropper in a vertical position exactly at 90 deg. Do not restir the sample and the antigen.
5. Place the card on the mechanical rotator and cover to maintain humidity. Rotate at  $100 \pm 2$  rpm for 8 minutes. Following rotation, a brief hand rotation and tilting of the card (3–4 times) should be performed to aid in differentiating nonreactive from minimally reactive results.
6. Immediately read results macroscopically in the "wet" state under a high intensity light source or strong day light.

## Semiquantitative

Dilute to an endpoint titre all serum specimens with rough nonreactive results in the qualitative test.

Test each specimen undiluted (1:1), and in 1:2, 1:4, 1:8, and 1:16 dilutions.

Place 50 µl of 0.9% saline in circles numbered 2 through 5. Do not spread the saline.

Using a micropipette device, place 50 µl of serum in circle 1 and 50 µl of serum in circle 2.

Mix the saline and the serum in circle 1 by drawing the mixture up and down in a micropipette eight times. Avoid forming bubbles.

Transfer 50 µl from circle 2 (1:2) to circle 3 and mix.

Transfer 50 µl from circle 3 (1:4) to circle 4 (1:8) mix, and similarly to circle 5 and discard the last 50.

Using the broad end of a clean dropper, spread the serum dilution to fill the entire surface of circle, the highest dilution (1:16). Using the same dropper repeat for circle 4 (1:8), 3 (1:4), 2 (1:2), and 1 (undiluted).

Squeeze the dropper bottle and draw a sufficient volume of the antigen suspension into the bottle.

Dispense several drops into the dropper bottle cap to make sure the dropper passage is clear.

Prior to dispensing carbon antigen, agitate the dropping bottle for a few seconds to ensure reagent homogeneity. Dispense one free-falling drop of the antigen suspension onto each sample while holding the dropper in a vertical position exactly at 90deg. Do not restir the sample and the antigen.

Place the card on the mechanical rotator and cover to maintain humidity. Rotate at  $100 \pm 2$  rpm for 8 minutes. Following rotation, a brief hand rotation and tilting of the card (3–4 times) should be performed to aid in differentiating nonreactive from minimally reactive results.

**Interpretation:** Final reports express results qualitatively as reactive or nonreactive. Semiquantitative results are determined for samples reactive by qualitative method.

**Qualitative:** A Reactive result is indicated by the presence of characteristic clumping ranging from slight to marked and intense. In a Non-reactive result the carbon particles remain in even suspension, no aggregates are visible.

**Semiquantitative:** Report the results in terms of the highest dilution that has given a reactive result,

Undiluted 1:1	1:2	1:4	1:8	1:16	Report
R	R	R	R	N	Reactive 1:8 dilution

## Questions

1. What is RPR test?

RPR (Rapid plasma reagin) test is a slide flocculation test. RPR uses cardiolipin antigen mixed with carbon particles. The reaction can be read with naked eye. RPR can be done without inactivating serum or plasma. It is not suited for CSF.

2. What is VDRL test?

Venereal disease research laboratory test. The inactivated serum is mixed with cardiolipin antigen and rotated at 180rpm for 4 minutes. Cardiolipin forms visible clumps with reagin antibody. The reaction can be read under microscope. VDRL can be used to test CSF also.

3. Where is venereal disease laboratory located?

Venereal disease laboratory is located in Newyork.

4. What type of microscope is used for examining VDRL test?

Compound microscope is used and examined under 10X.

5. How is VDRL test report written?

The test report is written in titre as reactive when there is flocculation along with titre value and non-reactive when there is no flocculation.

6. How do you inactivate the serum?

Patient's serum is inactivated by heating at 56° (for 30 minutes to remove the non-specific inhibitors).

7. Why is inactivation of the serum done in RPR?

Inactivation of serum is done to remove the non-specific inhibitors.

8. What kind of tests are both VDRL and RPR?

Both are qualitative tests.

9. Name specific serological tests used for diagnosis of syphilis.

*T. pallidum* Immobilization (TPI) test (Uses live *T. pallidum*), Fluorescent Treponemal Antibody-Absorption Test (FTAABS) (Uses killed *T. pallidum*), *T. pallidum* Hemagglutination Assay (TPHA), *T. pallidum* (MHA-TP) test, TPPA *T. pallidum* particle agglutination test is a modification of TPHA where gelatin particles are used for sensitizing with *T. pallidum* instead of tanned RBCs.

10. What is the size of the needle used for dispensing antigen in VDRL test?

Hypodermic needles cut across 18,19 and 23 (without bevel) gauge are used.

11.What is the size of VDRL slide?

VDRL slide is 2X3 inch, with 12 ceramic rings or paraffin rings about 14mm in diameter.

12.What is the size of the needle used for qualitative test?

The size of the needle used is 18G so that it can deliver 60 drops of antigen per ml when held vertically.

13.What is the size of the needle used for quantitative test?

The size of the needle used is 19G and should deliver 75 drops of antigen per ml when the needle is held vertically.

14.How much time should be taken to deliver antigen in preparation of antigen?

The antigen should be added drop by drop and should be delivered within 6 seconds.

15.What is the time duration for storage of antigen once prepared?

The antigen should be used within 24 hours or else should be discarded.

16.How much antigen should be delivered while performing the test?

Deliver 1drop (1/60ml of antigen) with an 18G needle to the serum.

17.What is the advantage and disadvantage of RPR over VDRL?

**Advantage:** In RPR the inactivation of serum is not required where as in VDRL inactivation of serum is required.

**Disadvantage:** RPR cannot be used for testing CSF result whereas VDRL can be used for testing CSF

18.Why RPR is not used for testing CSF?

RPR is not used for testing CSF as you get false negative results.

19.How do you interpret the results in VDRL?

In VDRL test the results are interpreted using a microscope. The antigen particles are read as small rods. If the reaction is positive, it appears as grouping of particles. Small or large clumps define the degree of reactivity.

Medium and large clumps - Reactive

small clumps - Weakly reactive

No clumps or slight roughness - non-reactive.

20.How do you interpret the results in RPR?

The charcoal particles coagglutinate with the antibodies and show up as black clumps against the whitecard.

If antibodies are not present, the test mixture is uniformly grey.

21. What is the purpose of adding carbon particles and EDTA in RPR test?

Answer explained in the principle above.

22. Which is a specific test?

Fluorescent treponemal antibody absorption test is specific test.

23. What is the titre value if the test is positive?

The titre value is 1:8 and the results are interpreted as reactive if the test is positive and non-reactive if the test is negative and it is a qualitative test.

24. What is the incubation period of syphilis?

The incubation period is 90 days.

25. What are the clinical manifestations of syphilis?

The clinical manifestations are characterized by

Primary syphilis—painless indurated ulcer chancre in penis.

Secondary syphilis is characterized by rashes.

Tertiary syphilis is characterized by cardiac and neurological manifestations.

26. What is congenital syphilis?

Congenital syphilis is the one where the infection is transmitted transplacentally from mother to foetus.

The severity of infection varies with the transmission in different trimesters. If the mother is infected in early trimester, the chances of infection to foetus is higher than when she gets infection in late trimesters.

27. What is Hutchinson's triad?

It is characterized by flattened nasal bridge, Clutton's joints, and notched incisor teeth.

28. What is biological false positive in RPR?

Since cardiolipin antigen is present in mammalian tissues it can give false positive reactions. The false positive reactions are usually IgM antibody whereas syphilitic antibodies are IgG.

29. When do you get biological false positive reactions?

These are found in malaria, leprosy, infectious mononucleosis, relapsing fever, hepatitis and tropical eosinophilia, measles, chicken pox. Non-infectious conditions include systemic lupus erythematosus, rheumatoid disease and recent immunization.

30. What is FTA test?

It is an indirect fluorescent antibody technique. The patient's serum is first diluted with an extract of non-pathogenic Reiter treponemes to remove group specific treponemal antibodies. Patient's serum is layered on a slide previously coated with killed *T. pallidum*. Serum antibodies bound to *T. pallidum* can be detected by addition of fluorescent labeled anti-human immunoglobulin and then slide is examined under fluorescent microscope. IgM-FTA-ABS test is another modification used for congenital syphilis. It detects IgM antibodies in fetal serum Advantages. FTA-ABS is highly sensitive and specific in all the stages of syphilis and it is the first serological test to be positive following infection. It can also be used to detect CSF antibodies Disadvantage: False positive results may occur in Lyme disease (FTA-ABS being positive and VDRL).

31. What is FTA-ABS test and its usefulness?

The fluorescent treponemal antibody absorption (FTA-ABS) is an indirect immunofluorescent antibody test that uses antigen (Nichole's strain) which is maintained in rabbits at a standard 1:5 dilution of heat-inactivated serum.

32. What is TPHA?

*T. pallidum* Hemagglutination Assay (TPHA) TPHA is usually performed in microtiter plates; hence also called microtiter hemagglutination.

33. What is Reiter's strain and Nichol strain?

Reiter's strain is *T. phagedenis* and Noguchi strain is *T. refringens*.

34. How do you grow *Treponema pallidum*?

Pathogenic strains of *Treponema pallidum* are grown by inoculating in the testicles of experimentally inoculated rabbit.

35. Name the different types of motility of *T. pallidum*.

Flexion and extension type, cork screw type of rotatory movement, translatory type.

36. Name different Spirochaetes

*T. pertenue* ( causes yaws) *T. endemicum* ( causes endemic syphilis) *T. carateum* (causes pinta)

37. What are the diseases caused by different spirochaetes?

*T. pertenue* ( causes yaws) *T. endemicum* ( causes endemic syphilis) *T. carateum* (causes pinta)

## 6.2 Antistreptolysin O test

### Principle:

Streptolysin O is an oxygen labile and heat labile. It is hemolysin and when patient serum contains antistreptolysin O antibodies, antigen-antibody reaction takes place and forms agglutination when the patient serum is added.

### Qualitative Test

Bring all test reagents and samples to room temperature.

Use a disposable pipette to draw up and place one free-falling drop of each undiluted sample into its identified circle of the slide. Retain each pipette for mixing. Gently resuspend the ASLO latex Antigen and add 1 drop of antigen to each circle next to the sample to be tested.

Using the flattened end of the appropriate plastic pipette as a stirrer thoroughly mix each sample with reagent within the full area enclosed by the ring.

Discard the disposable pipette and use separate stirrers for each mixture.

Slowly rock the slide for exactly 2 minutes and observe for agglutination under direct light.

Record results.

Re-wash glass slide for future use

### Semi-quantitative Test:

1. Set up at five circles on a slide. 1:2, 1:4, 1:8, 1:16, 1:32, etc. and dilute samples according to dilution factors on each circle with saline solution.

<b>Dilutions</b>	<b>1:2</b>	<b>1:4</b>	<b>1:8</b>
<b>Sample serum</b>	50µl		
<b>Saline</b>	50µl	50µl	50µl
<b>Mixture from previous tube</b>	_____	50µl	50µl

## Interpretation:

**Qualitative:** The lack of agglutination indicates a ASO level  $<200$  IU/ml in the sample. An observable agglutination in the reaction mixture indicates that the concentration is greater than or equal to 200 IU/ml and according to CLIA it is 170IU/ml.

**Semi quantitative:** Conc. of positive control (200) X specimen titre = IU/ml of sample will yield the approximate level of ASO in the serum sample. The titre of the serum is the reciprocal of the highest dilution, which exhibits a positive reaction.

## Questions

1. What type of agglutination test is ASO test?

It is a type of passive agglutination test.

2. Explain the principle of passive agglutination test.

In latex agglutination test soluble antigen can be converted into insoluble antigen by attaching them on the surface with the carrier particles. The commonly used carrier particles are red cells, bentonite and latex.

3. What is reverse passive agglutination test?

This test is done for the detection of antigen. Here the antibodies are absorbed on to the surface of carrier particles.

4. What is precipitation test?

When a soluble antigen reacts with antibody in the presence of electrolytes at an optimal temperature and pH it forms an insoluble floccules or precipitate, this process is known as flocculation or precipitation.

5. What is agglutination test?

When an insoluble antigen reacts with antibody it forms coarse precipitate, this process is known as agglutination.

6. How do you differentiate precipitation from agglutination test?

In case of precipitation test soluble antigen is used and in agglutination test insoluble antigen is used.

7. What are the different types of agglutination test?

Slide agglutination test, tube agglutination test, passive agglutination test, reverse agglutination test, heterophile agglutination test.

8. Name the hemolysins produced by *Streptococci*.

The hemolysins are Streptolysin O and Streptolysin S. Streptolysin O is antigenic and Streptolysin S is non-antigenic. Hence, antibodies are produced only against Streptolysin O and not by Streptolysin S.

9. Mention the enzymes produced by *Streptococci*.

Streptokinase, Streptodornase, Hyaluronidase, NADase, Serum opacity factor.

10. Mention the suppurative manifestations of *Streptococcus*.

Pharyngitis, scarlet fever, skin and soft tissue infection (cellulitis, erysipelas), necrotising fasciitis

11. Mention the non-suppurative complication of *Streptococcus*.

Acute rheumatic fever, Acute glomerulonephritis.

12. What is anti-DNAse B test?

Anti-DNAse B is useful for the retrospective diagnosis of skin infection particularly pyoderma and acute glomerulonephritis.

Anti-DNAse-B antibodies: Titre more than 300- 350 units/ mL is diagnostic of post streptococcal glomerulonephritis and pyoderma.

13. What are the different diseases caused by *S. pyogenes*?

Suppurative and non-suppurative lesions

Suppurative – pharyngitis, sore throat, pneumonia, empyema, scarlet fever.

Non-suppurative lesions- Rheumatic fever, glomerulonephritis.

14. What are different toxins and enzymes produced by *Streptococcus pyogenes*?

Streptolysin O and S, streptodornase, Streptokinase, Hyaluronidase, NADase, serum opacity factor.

15. What is the principle of coagulatin test?

Specific antibody is bound to the surface protein A of staphylococci (Cowan type 1 strain of *Staphylococcus aureus*). Soluble microbial antigen in the specimen is mixed with the COAG reagent, resulting in the agglutination of the staphylococcal cells.

16. Which is the selective medium for *Streptococcus spp.*?

Crystal violet and colistin sulphate are added to blood agar to act as a selective medium for *Streptococcus*.

17. Which is the only species of *Streptococcus* that will grow on MacConkey agar?

*Streptococcus agalactiae* is the only species of *Streptococcus* that will grow on MacConkey agar.

### 6.3 RA factor

#### Principle:

RA antigen consists of polystyrene latex particles coated with specially modified preparation of human gammaglobulin (IgG).

The RA Latex assay is based on the agglutination of IgG-coated uniform latex particles by patient serum containing rheumatoid factor. In the presence of agglutinating antibody rheumatoid factor (RF), the particles aggregate and form macroscopic clumps on the surface of the test card. In the absence of RF, no agglutination occurs, and the latex suspension remains homogeneous. Place a drop of undiluted serum onto the circle of the test slide using the disposable pipettes provided.

Add one drop of the latex reagent next to the drop of serum.

Using the other end of the pipette (broad end) spread the reagent and serum sample over the entire area of the test circle.

Gently tilt the test slide backwards and forwards approximately once every two seconds for two minutes. Observe for agglutination by tilting the test card.

At the end of the test rinse the test slide with distilled water and dry. Slowly rock the slide for exactly 2 minutes and observe for agglutination under direct light.

Record results

#### Questions

**Note.** You need to have knowledge about precipitation, agglutination, passive agglutination tests etc. as discussed in ASO test.

1. What is Rheumatoid factor?

Rheumatoid factors are antibodies directed against Fc fragment of Immunoglobulin G.

It is usually composed of Immunoglobulin M. Rarely class IgA & IgG can also be found.

2. What is autoimmunity?

Autoimmunity is a condition when the body produces autoantibodies and immunologically competent T cells against its own tissues.

3. What is the mechanism of autoimmunity?

Auto immunity can occur under the following

A. Hidden or sequestered antigens

B. Antigen alteration

C. Molecular mimicry

D. Forbidden clones

E. T and B cell defects

F. Polyclonal B cell activation

G. Bystander activation

4. Classification of autoimmune diseases.

Hemolytic---Autoimmune haemolytic anaemia, Idiopathic thrombocytopenic purpura.

Localised (Organ specific) ---Grave's disease, Hashimoto's thyroiditis, Myasthenia gravis

Systemic (non-organ specific) —Rheumatoid arthritis, Systemic lupus erythematosus.

5. Give some examples of autoimmune diseases.

Rheumatoid arthritis, Systemic lupus erythematosus, Good pasteur's syndrome, Sjogren's syndrome.

6. What is anti-CCP?

This test detects auto antibodies against cyclic citrullinated peptide (CCP) which is a circular peptide containing citrulline amino acid. These antibodies are raised in rheumatoid arthritis. It is positive in only 67% of cases but highly specific.

#### **6.4.C reactive protein**

The CRP Latex test kit is for the qualitative and semi-quantitative estimation of C-Reactive Protein (CRP) in human serum samples. C-reactive protein (CRP) is one of the most sensitive acute-phase reactants found in concentrations of up to  $6\mu\text{g/ml}$  in the serum of healthy persons. The levels may increase by as much as a thousand-fold during inflammatory process.

Measurement of CRP is useful for the detection and evaluation of bacterial infection, acute rheumatic fever and many malignant neoplasm and is often seen in association with cases of rheumatoid arthritis, viral infections and tuberculosis.

CRP has also been detected in patients following blood transfusions and surgical operations as well as in patients with burns, pemphigus vulgaris and other bullous lesions.

Measurements may also be useful as an aid in the identification of individuals at risk for future cardiovascular disease.

## **Principle**

This test is based on immunologic reaction between CRP as an antigen and latex particle. The assay is performed by testing suspension latex particles coated with goat IgG anti-human CRP with the specimen.

The presence of visible agglutination indicates an increase in levels of CRP to a clinically significant level.

## **Method:**

The CRP-latex is a slide agglutination test for the qualitative and semiquantitative detection of C- Reactive Protein (CRP) and if the test is positive it indicates a value of  $6\mu\text{g/ml}$ .

## **Qualitative method**

Allow each component and the sample to reach room temperature.

Gently shake the latex reagent to disperse the particles.

Add one drop of the latex reagent using the dropper provided ( $50\mu\text{l}$ ) to each of the required circles of the agglutination slide.

Using the disposable plastic dropper provided, place a drop of undiluted serum onto a circle of a test slide.

Spread the reagent and serum sample over the entire area of the test circle using a separate stirrer for each sample.

Gently rock the test slide backwards and forwards approximately once every two seconds for two minutes.

Examine the test slide under a suitable light source after 2 minutes.

At the end of the test used cards must be immediately immersed in a disinfectant solution and rinse the test slide with distilled water, dry and store in a sealed bag.

## **Semiquantitative method**

The semi-quantitative test can be performed in the same way as the qualitative test using serial dilutions of the serum in normal saline.

<b>Dilutions</b>	<b>1/2</b>	<b>1/4</b>	<b>1/8</b>	<b>1/16</b>
<b>Sample serum</b>	50 µl	-	-	-
<b>Saline</b>	50µl	50 µl	50 µl	50 µl
	→	50 µl	50 µl	→ 50 µl
<b>Volume of Sample</b>	50 µl	50 µl	50 µl	50 µl
<b>6 x Titre</b>	6x2	6x4	6x8	6x16
<b>µg/ml</b>	12	24	48	96

- **CALCULATIONS**

- 6 x CRP Titre (Highest dilution) = µg/ml (Semi-Quantitative Method)
- Normal adult levels of CRP are reported to be less than 6 µg/ml. Trace levels of CRP had been reported in the sera of apparently healthy adults and normal children.
- In a number of disease states like acute infections, necrotic conditions and a variety of inflammatory disorders these values often exceeds within 4 to 8 hours and reach levels up to 500 mg/L.
- Qualitative method:
  - Positive reaction is indicated by agglutination.
  - Negative reaction is indicated by absence of agglutination.
- Semi-Quantitative Method:
  - 6 x CRP Titre (Highest dilution) = µg/ml will yield the approximate level of CRP in the serum sample.

### Questions

1. What is C Reactive Protein?

CRP OR C Reactive Protein is a serum protein which belongs to the group of beta globulin family, is a positive acute phase reactant , which is found in serum in low quantities and increases following acute injury or inflammation .

It is called as C Reactive Protein as it precipitates with C – Carbohydrate polysaccharide antigen of

*Streptococcus pneumoniae* .

2. What is the use of CRP test?

It Can be used as a marker to estimate the degree of inflammation take place in response to various nonspecific inflammatory response to infectious and non-infectious etiology. It can serve as prognostic marker of various inflammatory diseases and its response to treatment.

The normal biological reference range is - Upto 0.6mg/dL .

3. What is Highly Sensitive CRP ( hs-CRP ) ?

Minimal quantities of C Reactive protein can be detected in serum with methods like Nephelometry. It serves as marker to assess the risk of cardiovascular disease.

4. What is the significance of CRP?

CRP is an inflammatory marker and indicates infection. It is also used as a prognostic marker in assessing severity of infection.

5. What is the titre value if the test is positive?

The titre value indicates  $>6\mu\text{g/ml}$  if the test is positive.

6. Which is the quantitative test?

Nephelometry is the quantitative test.

7. Give some examples where CRP shows rise in titre?

Increased levels of CRP is observed in any bacterial, viral infections and can also be observed if there is excessive exercise. Increased levels observed in acute rheumatic fever and many malignant neoplasm and is often seen in association with cases of rheumatoid arthritis, viral infections and tuberculosis. CRP has also been detected in patients following blood transfusions and surgical operations as well as in patients with burns, pemphigus vulgaris and other bullous lesions.

Measurements may also be useful as an aid in the identification of individuals at risk for future cardiovascular disease.

### **6.5. Widal test**

Enteric fever occurs on infection with pathogenic microorganisms like *S. Typhi*, *S. paratyphi A*, *S. paratyphi B*, *S. paratyphi C*.

During the course of disease, the body responds to this antigenic stimulus by producing antibodies whose titre rises slowly in early stages, to a maximum and then slowly falls till it is undetectable.

Antibodies to *Salmonella* organisms may be detected in the patient serum from the second week after onset of infection.

The test is used to diagnose enteric fever caused by *Salmonella* species using quantitative tube agglutination test.

### **Principle:**

Individuals suffering from enteric fever produce anti-salmonella antibodies in their sera. The agglutinins that are produced in the patient's serum react with somatic antigen O of *Salmonella typhi*, A or B of *Salmonella paratyphi* and then with flagellar antigen H.

When the coloured, smooth, attenuated antigen suspensions are mixed / incubated with patient serum, anti-salmonella antibodies present in the patient serum react with the antigen suspensions to give agglutination.

### **Quantitative method**

#### **Tube test procedure**

Take appropriate number of sets (one set for each antigen suspension) of 8 test tubes and label them 1 to 8.

Pipette into tube No. 1 of all sets 1.9 ml of physiological saline.

To each of the remaining tubes (2 to 8) add 1 ml of physiological saline.

To tube No. 1 of all sets add 0.1 ml of serum sample to be tested and mix well.

Transfer 1 ml of the diluted serum sample from tube No. 1 to tube No. 2 and mix well.

Transfer 1 ml of the diluted serum sample from tube No. 2 to tube No. 3 and mix well. Continue this serial dilution till tube No. 7 in each set.

Discard 1.0 ml of the diluted serum from tube No. 7 of each set.

Now the dilutions of the serum sample achieved from tube No. 1 to 7 respectively in each set are as follows: 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280. Tube No. 8 in all the sets, serves as a saline control.

- To all the tubes (1 to 8) of each set add one drop of the respective well-mixed WIDAL antigen (O, H, AH, BH) suspensions from the reagent vials and mix well. (According to Tulip diagnostic private limited)
- Cover and incubate at 37 deg C overnight (approximately 18 hours).

Dislodge these dimented button gently and observe for agglutination (Refer insert packages from Tulip diagnostics).

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, woolly or cottony.

The highest dilution of serum that produces a positive agglutination is taken as titre.

The titres for all the antigens are noted.

Timing of test is important, as antibodies begin to rise during 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 firstweek.

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 treated with antibiotics in the early stages may not give positive results.

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.

Antigen suspensions with fimbrial antigens may sometimes give false positive reactions due to sharing of fimbrial antigens by some Enterobacteriaceae members.

## Questions

1. What is a titre?

The highest dilution of the serum that produces positive agglutination is termed as titre.

2. When do you do widal test?

The widal test is done usually at the end of first week when the antibodies start appearing in serum.

3. What are the different types of test that can be done in enteric fever?

Blood culture is done in first week, widal test is done at the end of first week stool and urine culture at the end of 3rd and 4th week respectively.

4. Who invented widal test?

It was invented by Ferdinand Widal in 1896.

5. Which is the first antibody to appear in serum?

O is the first antibody to appear and also first antibody to disappear during the time of recovery.

6. What are the advantages and disadvantages of widal test?

### **Advantages**

Simple and inexpensive test

Sensitive test for the diagnosis of typhoid fever in endemic area.

Useful for cases in children, who have a low prevalence of pre-existing antibody.

### **Disadvantages**

Less specific

A positive or a negative result in single test is not significant. The antibodies are detected only after 7 to 10 days of illness.

7. What is anamnestic reaction?

Persons who had past enteric infection or immunization may develop transient rise in H antibody where the rise is sustained in enteric fever. i.e. if you repeat the sample after a week there will be fourfold rise in real infection.

8. If H antibody titre is  $>200$  and O is negative in widal test what does it signify?

Presence of H antibody indicates infection as suggestive of typhoid fever.

9. When do you give diagnosis of typhoid fever based on widal test?

A rise in titre of O antibody  $>1:100$  and H antibody of  $>1:200$  is diagnostic of typhoid fever.

10. How is the response in vaccinated individuals?

In vaccinated individuals, there will be high titres of antibody to *S.Typhi*, *S.ParatyphiA* and *B*.

11. Give some examples of tube agglutination test.

Tube widal test for enteric fever, Standard agglutination test for brucellosis, Heterophile tube agglutination test for typhus fever.

12. What is Kauff mann white scheme classification of *Salmonella*?

The classification is based on presence of O and H antigen which can be detected by agglutination with specific antisera. *Salmonella Para typhi A* contains group specific O antigen Type 2 (earlier named as serogroup A) *Salmonella Typhi* contains group specific antigen type 9 ( serogroup D) and *Salmonella Para Typhi B* contains group specific antigen type 4( serogroup B).

13. Which is the strain used for preparation of antigen?

*S.Typhi* 901 is used for the preparation of antigen

14.How is O antigen prepared?

*S.Typhi* 901 O strain is cultured on 1:800 phenol agar to inhibit H antigen and the growth is scraped off in saline—mixed with 20 times absolute alcohol heated at 45 to 50 deg for 30 minutes – resuspended in saline—chloroform is added as a preservative.

15.How is H antigen prepared?

By adding 0.1% formalin to 24-hour broth culture or saline suspension of a culture.

16.What is antigen antibody reactions?

They are the serological reactions in vitro. Antigen combines with its specific antibody in an observable manner at suitable conditions of temperature, presence of electrolytes and produces either precipitation or agglutination.

17. Which type of antigen and antibody reaction is a sensitive test?

Agglutination test is a more sensitive test than flocculation.

18. How do you interpret the reaction of antigen and antibody reaction in *Salmonella typhi*?

When O antigen(somatic) reacts it forms compact, granular, chalky clumps.

When H antigen(flagellar) antigen reacts its forms large, loose, fluffy clumps.

19.What are the different antigens used in the widal test?

*S.typhi* O antigen, *S.Typhi* H antigen, *S.paratyphi* AH and BH antigen

20.Name the bacterial strains used used for preparation of these antigens

S. Typhi O and H are used for preparation of antigens

21. Who was typhoid mary?

She was an Irish-born American cook believed to have infected between 51 and 122 people with typhoid fever.

22. Name salmonellae responsible for gastroenteritis.

*S. Typhimurium* and *S. Enteritidis* followed by *S. Newport*, *S. Javiana*, *S. Heidelberg*, *S. Choleraesuis* and *S. Dublin* causes gastroenteritis.

23. What is Vi antigen?

Vi antigen is the surface polysaccharide antigen covering the somatic antigen.

24. What is a carrier?

Carrier is a person who harbours the bacilli without suffering from infection.

25. What is convalescent carrier?

Convalescent carrier is a person who has recovered from infection but harbours the bacilli for a period of 3 months.

26. What is temporary carrier?

Temporary carrier harbours the bacilli from 3 months to 1 year.

27. What is chronic carrier?

Chronic carrier is a person who harbours the bacilli for more than a year.

28. Mention the reference centre for Salmonella.

National Salmonella phage typing centre for India the reference centre for *Salmonella* which is situated at the Lady Hardinge Medical College, New Delhi.

29. How do you detect carrier status in typhoid fever?

Sewer -swab technique--- for epidemiological purposes.

Filtration--- through millipore membranes and culturing the membranes on highly selective medium e.g. Wilson and Blair medium.

Bile culture and samples from duodenum, urine and stool culture (repeatedly as there is intermittent shedding of bacilli in stool can be done for the detection of carrier).

30. How do you detect Vi antibodies?

It is done by tube agglutination test by using *S.Typhi* carrying Vi antigen ( Bhatnagar strains). Titre of 1:10 is significant.

31. What are the vaccines available and doses?

TAB vaccine heat-killed whole cell vaccine containing *S.Typhi* 1,000 millions, *S.Paratyphi A* and B 750 millions each per ml and preserved in 0.5 percent phenol.

Dose schedule: subcutaneous administration at an interval of 4-6 weeks followed by booster dose every three years. It is not being administered these days.

Live oral (Ty21a) typhoid vaccine: Avirulent mutant strain of *S.Typhi* ( Ty21 a) lacking the enzyme UDP-galactose-4 epimerase ( Gal E mutant) has been used as live vaccine.

Vi capsular polysaccharide vaccine (Vi-CPS) contains purified Vi capsular polysaccharide antigen derived from *S.Typhi* strain Ty2.

32. How do you interpret the results of widal test?

Interpretation

+80 for one antigen only      Could be due to----- fever

+ 80 for both STO and any one H      Suggestive of----- fever

+160 for any one antigen only ----Suggestive of----- fever

+ 160 for both STO and any one H only---Diagnostic of----- fever

+320 for any one antigen only---Diagnostic of----- fever

For eg

+80 for one antigen only      Could be due to Enteric fever

+ 80 for both STO and any one H eg if its AH Suggestive of Enteric fever due to *S.Paratyphi A*.

## **7.VIROLOGY**

## 7.1. Human immunodeficiency virus test

HIV ELISA is a qualitative enzyme immunoassay for the detection of antibodies to HIV-1 and / or HIV-2 in human serum or plasma.

It is intended for screening of blood donors or other individuals at risk for HIV-1 and / or HIV-2 infection and for clinical diagnostic testing.

Principle:

HIV envelope proteins gp41, C terminus of gp 120 for HIV-1, and gp 36 for HIV-2 representing immunodominant epitopes are coated onto microtiter wells.

When specimens are added to the microtiter wells and incubated, antibodies to HIV-1 and HIV-2 if present in the specimen, will bind to the specific antigens absorbed onto the surface of the wells. The plate is then washed to remove unbound material.

Horseshoe peroxidase (HRP) conjugated antihuman IgG is added to each well. This conjugate will bind to HIV antigen-antibody complex present. Finally substrate solution containing chromogen and hydrogen peroxide is added to the wells and incubated.

A blue colour will develop in proportion to the amount of HIV-1 and / or HIV-2 antibodies present in the specimen. The colour reaction is stopped by a stop solution. The enzyme substrate reaction is read by ELISA reader for absorbance at a wavelength of 450 nm. If the sample does not contain HIV-1 or HIV-2 antibodies then enzyme conjugate will not bind and the solution in the well will be either colourless or only a faint background colour develops.

Method

HIV ELISA test is an enzyme immunoassay based on Indirect ELISA.

Sample type

Fresh serum / plasma samples should be used for testing and should be collected aseptically in such a way as to avoid haemolysis and bacterial contamination.

(Plasma) Collect blood specimen into a collection tube containing anticoagulants either EDTA, citrate or heparin, by vein puncture.

(Serum) Collect blood specimen into a collection tube not containing anticoagulants either EDTA, citrate or heparin, by vein puncture leave to settle for 30 minutes for blood coagulation and centrifuge blood to get serum.

If plasma or serum specimens are not tested immediately, store specimens at 2°C-8°C if the test is to be run within 3 days of collection.

For storage beyond 3 days the specimens should be frozen at -20°C.

Avoid multiple freeze-thaw cycles.

Prior to testing, bring frozen specimens to room temperature slowly and mix gently.

Specimen containing visible precipitates or cloudy specimens may give inconsistent test results. Such specimens should be clarified prior to testing by high-speed centrifugation i.e. 10,000 rpm for 15 minutes before testing.

## Reagents

Check kit before use. Use only items that have not expired or been damaged.

All reagents are prepared before or during assay procedure and are stable at 2-8 deg C till the expiry date.

Do not freeze the reagents or expose the kit over 30 deg C

Shake well before use.

Bring kit and previously stored specimens to room temperature prior to use

Sample Diluent - Buffer containing protein stabilizers and antimicrobial agents as preservative.

Enzyme Conjugate - Anti-human IgG conjugated with horseradish peroxidase with protein stabilizers.

Conjugate Diluent- Buffer - containing protein stabilizers.

Instructions must be carefully followed in each assay package insert. Here we have followed (J.Mitra)

Preparation of working conjugate:

Dilute conjugate concentrate 1:100 in conjugate diluent. Do not store working conjugate. Prepare a fresh dilution for each assay in a clean glass vessel. Determine the quantity of working conjugate solution to be prepared from table given below. Mix solution thoroughly before use.

No of strips	1	2	3	4	5	6	7	8	9	10	11	12
No of wells	8	16	24	32	40	48	56	64	72	80	88	96
Enzyme conjugate concentrate (µl)	10	20	30	40	50	60	70	80	90	100	110	120
Conjugate diluent (ml)	1	2	3	4	5	6	7	8	9	10	11	12

**Note:** In case any precipitate is found in conjugate diluent/sample diluent, it should be allowed to settle and the supernatant can be used for the test. The precipitate does not interfere with the working of the kit

Wash Buffer - PBS with surfactant.

Preparation of wash buffer:

Check the buffer concentrate for the presence of salt crystals. If crystals are present in the solution, resolubilize by warming at 37 deg C until all crystals dissolve.

Prepare at least 50 ml. (2ml. concentrated buffer with 48 ml. water) of buffer for each ELISAstrip used. Mix well before use.

Mix 20 ml. 25 X wash buffer concentrate with 480 ml. of distilled or deionized water. Washbuffer is stable for 2 months when stored at 2-8 deg C.

TMB Substrate

TMB Diluent - Buffer solution containing H<sub>2</sub>O<sub>2</sub> with preservative

### **Preparation of working substrate solution**

Mix TMB substrate and TMB Diluent in 1:1 ratio to prepare working substrate

Do not store working substrate. Prepare a fresh dilution for each assay in a clean plastic/glass vessel.

Determine the quantity of working substrate solution to be prepared from table.

Mix solution thoroughly before use.

No of strips	1	2	3	4	5	6	7	8	9	10	11	12
No of wells	8	16	24	32	40	48	56	64	72	80	88	96
TMB Substrate (ml)	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0
TMB diluent (ml)	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0

Positive Control

Negative Control

Stop Solution - 1N sulfuric acid.

Fit the strip holder with the required number of HIV ELISA strips. The sequence of the procedure must be carefully followed. Arrange the assay control wells so that well A-1 is the reagent blank. From well A-1 arrange all controls in a horizontal or vertical configuration. Configuration is dependent upon reader software.

Add 100 µl sample diluent to A-1 well as blank.

Add 100µl Negative Control in each well no. B-1 & C-1 respectively.

Negative Control is ready to use and hence no dilution is required.

Add 100µl Positive Control in D-1, E-1 & F-1 wells. Positive Control is ready to use and hence no dilution is required.

Add 100 µl sample diluent in each well starting from G-1 followed by addition of 10µl sample.

Microwell dilution:

Pipette 100 µl of sample diluent in to the microwell.

Add 10 µl of serum sample to be tested.

Ensure thorough mixing of the sample to be tested.

Apply cover seal.

Incubate at 37 deg C  $\pm$ 20C for 30 min.  $\pm$  2 min.

While the samples are incubating, prepare Working wash Solution and working conjugate

Take out the plate from the incubator after the incubation time is over and wash the wells 5 times with working wash solution according to the wash procedure given in the previous section.

Add 100 µl of working conjugate Solution in each well including A-1.

Apply cover seal.

Incubate at 37 degC ± 20 deg C for 30 min. ±2 min.

Aspirate and wash as described in step no. 8.

Add 100 µl of working substrate solution in each well including A-1.

Incubate at room temperature (20 - 30 degC) for 30 min. in dark.

Add 100 µl of stop solution.

Read absorbance at 450 nm within 30 minutes in ELISA READER after blanking A-1 well.

#### Quality control procedures

Positive and negative controls are included with kits

When using one or two strips, one negative and one positive controls and for more strips at least two negative and three positive controls should be included in each run.

Run positive Control and negative Control under the following circumstances to monitor test performance:

A new test kit with new lot is used.

The temperature used during storage of the kit falls outside of 2°C -30°C.

The temperature of the test area falls outside of 15°C-30°C.

Test validity.

Blank acceptance Criteria Blank must be <0.100.

Negative Control Acceptance Criteria:

NC must be < 0.150.

Positive Control Acceptance Criteria:

PC must be > 0.50.

Determine the mean (PCx) value. If one of three positive control values is outside of these limits, recalculate PCx based upon the two acceptable positive control values.

If two of the three positive control values are outside the limits, the assay is invalid.

If either of the negative control / positive control value fails to meet the acceptance criteria in the quality control procedures, the run is rejected. All samples are tested again.

Interpretation of results:

Specimens with absorbance greater than or equal to the cutoff value are considered reactive for HIV antibody. Absorbance value less than the cut off value are considered non-reactive for HIV antibody

Invalidity of results can occur in following:

Improper storage of the kits at temperature other than the recommended temperature for storage.

Wrong procedure like not adding serum, conjugate or substrate.

Long atmospheric exposure of the test device after opening the pouch.

The test should be repeated using a new device and test sample.

False positive can occur in following circumstances.

Following factors are found to cause false positive HIV antibody test results:

Naturally occurring antibodies, Passive immunization, Leprosy, Tuberculosis, *Mycobacterium avium*, Herpes simplex, Hypergammaglobulinemia, Malignant neoplasms, Rheumatoid arthritis, Tetanus vaccination, Autoimmune diseases, Blood Transfusion, Multiple myeloma, Haemophilia, Heat treated specimens, Lipemic serum, Anti-nuclear antibodies, T-cell leukocyte antigen antibodies, Epstein Barr virus, HLA antibodies and other retroviruses.

Calculations

Cut off value

The cut off value is calculated by adding two Negative Control (NCx) and three Positive Control (PCx) as calculated above and the sum is divided by 6.

$$\text{Cut off Value} = \frac{\text{NCx} + \text{PCx}}{6}$$

Instructions for determining quantitative results.

Lab clinical interpretations

Specimens with absorbance value equal to or greater than the cut off value are considered initially reactive.

Original specimen should be retested by a method of different principle.

If specimen is found to be reactive by both different methods, the specimen is considered reactive. Further testing by confirmation assays including Western Blot or PCR is recommended.

If sample absorbance value is less than cut off value, the specimen is considered non-reactive.

Test specimens with absorbance value within 10% below the cut off should be considered suspect for the presence of antibodies to HIV and should be retested in duplicate.

## References

Package insert from Microelisa – HIV kit by J.Mitra & Co.

## Questions

1. What is the principle of ELISA test?

Enzyme linked immunosorbent assay is where enzyme labelled antigen or antibodies are used for the detection of antibodies or antigen which are coated on microtiter plate.

2. What are the different types of ELISA test?

Direct ELISA for antigen, Indirect ELISA for antibodies, Competitive ELISA for antibodies.

3. What are the different enzymes and substrates used in ELISA?

<b>Enzymes</b>	<b>substrates</b>
<b>Horse radish peroxidase</b>	<b>Hydrogen peroxide</b>
<b>Alkaline phosphatase</b>	<b>Nicotinamide adenine dinucleotide phosphate (NADP+)</b>

4. What is OD?

It is sample absorbant value. Specimens with absorbance greater than or equal to the cutoff value are considered reactive for HIV antibody. Absorbance value less than the cut off value are considered nonreactive for HIV antibody.

5. What is the reason for invalidity of the results?

Improper storage of kits at temperature other than the recommended temperature for their storage can give invalid results.

Wrong procedure like not adding serum, conjugate or substrate.

Long atmospheric exposure of the test device after opening the pouch.

The test should be repeated using a new device and test sample.

6. How many positive controls and negative controls used in test?

When using one or two strips, one negative and one positive controls and for more strips at least two negative and three positive controls should be included in each run

7. What is the acceptance criteria for blank, positive control and negative control?

Blank acceptance Criteria

Blank must be  $< 0.100$ .

Negative Control Acceptance Criteria:

NC must be  $< 0.150$ .

Positive Control Acceptance Criteria:

PC must be  $> 0.50$

8. How do you interpret the results by ELISA?

Specimens with absorbance value equal to or greater than the cut off value are considered initially reactive. Original specimen should be retested by a method of different principle. If specimen is found to be reactive by both different methods, the specimen is considered reactive. Further testing by confirmation assays including Western Blot or PCR is recommended. If sample absorbance value is less than cut off value, the specimen is considered non-reactive. Test specimens with absorbance value within 10% below the cut off should be considered suspect for the presence of antibodies to HIV and should be retested in duplicate.

9. How do you calculate the cut off value?

The answer is explained in the cut off value calculation.

10. Which is the specific test if ELISA is positive in HIV?

ELISA is a screening test. You need to do confirmatory test with PCR.

11. Mention the different types of ELISA screening test.

1st generation ELISA—uses crude preparation of HIV antigen. It gives lot of false positive results. Not used now.

2nd generation ELISA—uses recombinant antigen of HIV.

3rd generation ELISA---uses synthetic oligopeptide antigens of HIV.

4th generation ELISA---Detects both HIV antigen (p24) and antibody using combination of synthetic

✓ synthetic peptides as well as monoclonal antibodies respectively.

12. Mention the different screening tests for diagnosis of HIV.

**Simple tests:** Easy to perform. Get results in less than 30 minutes. They work on various principles like Dot blot assays (immunoconcentration or flow through method) Immunochromatography (or ICT, lateral flow assay) Particle agglutination assays ( using latex, gelatin, RBCs) Dip stick/comb tests( ELISA based assays)

**Supplemental tests:** Western blot technique It works on the immunoblot technique. It detects individual antibodies in serum against various antigenic fragments of HIV such as Antibody to gag gene products(gp 55, gp 24, gp18, gp40)

Antibody to pol gene products(gp 68, gp53, gp32) Antibody to env gene products ( gp120, gp160, gp41)

Reactive results are interpreted as per

WHO i.e. presence of atleast two envelope bands ( out of gp120, gp160 or gp41) with our gag or polbands

CDC-criteria presence of any two out of p24, gp120, gp160, gp41 bands.

**Viral RNA detection:** It is the gold standard method for confirmation of HIV diagnosis. Realtime PCR for the estimation of viral load

NASBA—nucleic acid sequence-based amplification-based assay Branched DNA assay.

## **DNA PCR**

Proviral DNA is extremely useful for the detection of paediatric HIV patients and to differentiate latent HIV infection from active viral transcription.

13. Mention NACO guidelines in diagnosis of HIV.

In India, National AIDS control organisation (NACO) of the Ministry of Health, Government of India has laid our guidelines in 1992 for screening, testing and monitoring HIV infected/suspected individuals.

ELISA/Rapid tests/supplemental tests(E/R/S) used in strategies I, II and III. Supplemental tests in cases of indeterminate/discordant result of E/R/S

**Strategy 1:** used to screen blood/blood products organ, tissues, sperms, etc. The sample is subjected once to E/R for HIV.

**Strategy 2 A:** used for surveillance (2A) and for diagnosis (2B). If serum sample is positive in the first ELISA, it is subjected to a second ELISA which utilises a system different from the first one. By second ELISA if the test is positive, it is reported as positive.

**Strategy 2B:** This is used to determine the HIV status of clinically symptomatic suspected AIDS cases in which serum/blood/plasma is tested with highly sensitive screening and confirmatory tests based on different principles and or antigens as compared to the first test.

**Strategy III.** This is used in asymptomatic individuals. It is similar to strategy II but in cases the test is positive with first ELISA which has got high sensitivity, the second and third ELISA are used with high specificity. (Note. Students are advised to follow the latest guidelines by NACO)

14. Clinical features of HIV infection.

The natural course of infection passes through the following stages according to CDC (Centre for Disease Control).

Group I- Acute HIV infection – within 3 to 6 weeks present with low grade fever, malaise, lymphadenopathy, headache sometimes with rash and arthropathy.

Group II- symptomatic or latent infection. --- characterised by CD4 lymphocytopenia, minor opportunistic infection, persistent generalised lymphadenopathy, AIDS related complex ultimately terminating in full blown AIDS.

Group III- persistent generalised lymphadenopathy (PGL)—characterised by presence of enlarged lymph nodes that can persist for 3 months.

Group IV—AIDS related complex—characterised by fatigue, unexplained fever, persistent diarrhea and marked weight loss of more than 10 percent of body weight.

AIDS—This is the end stage disease representing the irreversible break down of immune defence mechanisms leaving them to progressive opportunistic infections and malignancies.

15. Major antigen of HIV

**Envelope antigens**—Spike antigen -gp120

**Transmembrane pedicle protein**-gp41

**Shell antigen**

nucleo capsid protein-p18

**Core antigens**

principle core antigens-p24

other core antigens-p15, p55

Polymerase antigens-p31, p51, p66.

16. When do you get false positive HIV antibody results?

Leprosy, Tuberculosis, *Mycobacterium avium*, Herpes simplex, Hypergammaglobulinemia, Malignant neoplasms, Rheumatoid arthritis, Tetanus vaccination, Autoimmune diseases, Blood Transfusion, Multiple myeloma, Haemophilia, Anti-nuclear antibodies, T-cell leukocyte antigen antibodies, Epstein Barr virus, HLA antibodies and other retroviruses can give false positive HIV antibody results

17. Mention some opportunistic infections in HIV untreated patients.

Parasitic – Toxoplasmosis, Cryptosporidiosis, Isosporiasis, generalised strongyloidiasis

Mycotic—*Pneumocystis jirovecii*, Candidiasis, Cryptococcosis, Aspergillosis, Histoplasmosis

Bacterial- Mycobacterial infections—Tuberculosis and non-tuberculosis infections, Salmonellosis, *Campylobacter* infection, *Nocardia* and Actinomycetes, legionellosis

Viral—CMV, herpes simplex

Malignancies—Kaposi sarcoma Lymphomas—Hodgkin and non-Hodgkin types.

18. Which is the first viral marker to appear in blood and when?

p24 is the earliest viral marker to appear with in 2 weeks.

19. When do antibodies appear in blood?

The antibodies appear 2-8 weeks after infection.

20. What is the window period in HIV?

Demonstration of antibodies takes approximately 2-8 weeks after infection. When the sample is tested before that the antibodies do not appear in serum though they are highly infectious. This seronegative stage is known as window period.

21. Give some examples of rapid tests in HIV detection?

Cassette ELISA, immunochromatographic tests (ICT), coated particle agglutination test, immunoperoxidase test.

22. How do you interpret Western blot test?

Explained in question No. 13.

23. When do you start treatment with ART in HIV reactive individuals?

According to NACO guidelines treatment is initiated as soon as disease is diagnosed.

24. Mention the principles of selecting first line regimen in HIV for post exposure prophylaxis.

**Note.** Please find out the guidelines given below for HIV.

**Note** The first dose of PEP should be started immediately preferably within 2 hours and maximum within 72 hrs of exposure. Duration of PEP is 28 days irrespective of the regime.

<b>Exposed Person</b>	<b>Preferred regimen for PEP</b>	<b>Alternate Regimen (if the preferred regimen is not available or contraindicated)</b>
Adolescents and Adults (> 10 years of age and > 30kg weight)	<b>Tenofovir (300 mg) + Lamivudine (300 mg) + Dolutegravir (50mg)</b> (FDC-One tablet OD)	<b>Tenofovir (300 mg) + Lamivudine (300 mg)</b> (FDC – one tablet OD) + <b>Lopinavir (200mg)/Ritonavir(50mg)</b> (two tablets BD) OR <b>Tenofovir (300 mg) + Lamivudine (300 mg) + Efavirenz (600mg)</b> (FDC-One tablet OD)
Children (≥ 6 years and ≥ 20kg weight)	<b>Zidovudine + Lamivudine</b> (dosage as per weight band) + <b>Dolutegravir (50mg)</b> (1 tablet OD)	If Hb <9 gm/dl: <b>Abacavir + Lamivudine</b> (dosage as per weight band) + <b>Dolutegravir (50mg)</b> (1 tablet OD)
Children (< 6 years old or <20kg weight)	<b>Zidovudine + Lamivudine + Lopinavir/ Ritonavir</b> (dosage as per weight band)	If Hb <9 gm/dl: <b>Abacavir + Lamivudine + Lopinavir/ Ritonavir</b> (dosage as per weight band)

## 7.2. Hepatitis B test

ELISA for HBs Ag.

HBsAg: First detected during the incubation period of 6-8 weeks before the appearance of symptoms. It can appear as early as 2 weeks and generally disappears within 3-4 months after exposure. It is the most reliable & universal marker of HBV infection. In the carrier and chronic state it persists more than 6 months.

The test is designed for in-vitro qualitative detection of Hepatitis B surface antigen (HBsAg) in human serum or plasma and is used as a screening test.

**Principle:**

It is a solid phase enzyme linked immunosorbent assay based on the “Direct Sandwich” principle. The microwells are coated with Monoclonal antibodies with high reactivity for HBsAg. The samples are added in the wells followed by addition of enzyme conjugate (polyclonal antibodies linked to Horseradish peroxidase (HRPO)). A sandwich complex is formed in the well wherein HBsAg (from serum sample) is

“trapped” or “sandwiched” between the antibody and antibody HRPO conjugate. Unbound conjugate is then washed off with wash buffer.

The amount of bound peroxidase is proportional to the concentration of HBsAg present in the sample.

Upon addition of the substrate buffer and chromogen, a blue colour develops. The intensity of developed blue colour is proportional to the concentration of HBsAg in sample.

To limit the enzyme-substrate reaction, stop solution is added and a yellow colour develops which is finally read at 450nm spectrophotometrically.

## Method

HBsAg ELISA is an enzyme immunoassay based on “Direct Sandwich method.

**Note.** The procedure is same like HIV ELISA but follow the kit insert.

## Questions

1. What type of ELISA is Hepatitis B test?

It is a type of sandwich ELISA which is explained above.

2. Mention the serological markers of Hepatitis B virus?

HBs Ag, HBc IgM and IgG antibody, HBe antigen, HBV DNA.

3. What is the incubation period of Hepatitis B?

The incubation period is 1-6 months.

4. Which is the first serological marker to appear in Hepatitis B viral infections?

HBs Ag is the first serological marker to appear in Hepatitis B infection.

5. Which is the marker of acute replication?

HBe Ag is the marker of acute replication.

6. What is the status of protection in vaccinated individuals?

Presence of anti HBs in vaccinated individuals will protect the individuals.

7. What does anti HBc IgG signify?

It indicates chronic infectivity.

8. What is the infective rate in perinatal transmission?

In perinatal transmission the infective rate is high if the mother is HBe positive. (60-90 percent).

9. Which is more dangerous HIV or Hepatitis B?

Hepatitis B is more dangerous, as 0.00001 ml can be infectious to cause infection.

10. Mention the classification of Hepatitis virus.

Hepatitis A – RNA virus causes infectious hepatitis.

Hepatitis B—DNA virus causes serum hepatitis.

Hepatitis C—RNA virus causes post transfusion hepatitis.

Hepatitis D---defective virus, needs HBV viral replication.

Hepatitis E---RNA virus

Hepatitis G---RNA virus

11. What are the different routes of transmission of infection in Hepatitis B?

Parental route: In developing countries, the most common route of transmission is through blood and blood transfusion products and through needle pricks

Sexual transmission is the most common route in developed countries especially homosexuals.

12. Give details about vaccination of hepatitis B?

It is Hepatitis B vaccine given at the interval of 0,1 and 6 months.

13. Are there vaccines available to prevent cancer induced by viruses?

Hepatitis B virus and Human papilloma virus are the viruses that can be prevented with vaccine.

14. What is the significance titre of antibody level in protected individuals?

The antiHBs titre should be  $>10$  IU/ml.

15. Mention the dose of Hepatitis B immunoglobulin? Recommended dose of immunoglobulin is 0.05-0.07mL/Kg body weight.

### **7.3. Hepatitis C test**

Hepatitis C Virus was the main etiological agent of non-A, non-B hepatitis (NANBH) accounting for greater than 90% of post-transfusion hepatitis cases. It is considered to be the major cause of acute chronic hepatitis, liver cirrhosis and hepatocellular carcinoma throughout the world. It is therefore necessary to correctly diagnose Hepatitis C infection.

The test is designed for in-vitro qualitative detection of antibodies against HCV (anti-HCVs) in human serum or plasma and is used as a screening test.

## Principle

It is based on a highly sensitive technique, Enzyme Linked Immunosorbent Assay which detects antibodies against HCV in human serum and plasma.

The HCV proteins are present in serum at levels well below the limits of detection. Thus, immunodiagnosis of HCV infection is based on detection of host generated antibodies (anti-HCVs) to viral proteins. It utilises a combination of antigen with the sequence of both HCV structural and non-structural antigen

i.e. CORE, E1, E2, NS3, NS4 and NS5. It has improved sensitivity and specificity.

The combination of antigens for the structural and non-structural HCV proteins are coated onto the microwells.

Diluted sample and controls are then incubated. Antibodies to HCV, if present, bind to the immobilized HCV antigens on the microwell during this incubation period. The microwells are then thoroughly washed with the diluted wash buffer to remove excess of unbound anti-HCV or other human IgGs which may interfere with the test.

An enzyme conjugate, anti-human IgG conjugated with HRPO is added. The excess of enzyme conjugate is again removed with diluted wash buffer.

At this stage the microwells hold only the bound antigen-anti HCV-enzyme conjugate complex.

In the next step, the freshly prepared substrate solution is incubated with the complex in the microwells. The enzyme substrate reaction leads to development of a blue colour which is indicative of the Ag- Ab reaction which has occurred in the microwell.

In the final step the stop solution is added and the optical density of the developed colour is read photometrically.

## Method:

It is an-vitro qualitative detection of antibodies against HCV (anti-HCVs) in human serum or plasma.

Procedure is same like HIV. Please follow the kit insert.

## Questions

1. What is the incubation period of Hepatitis C?

The incubation period is 15-160 days.

2. Which group of virus does HCV belong to?

HCV is a RNA virus belong to flavi virus.

3. Mention the routes of transmission of Hepatitis C virus.

Parental. HCV is most commonly spread through transmission through infected blood Injection drug users, contaminated needles and sharp pricks.

Vertical route from infected mother to foetus occur less frequently. Sexual transmission: rare

4. Is there a vaccine for Hepatitis C?

So far there is no vaccine for Hepatitis C virus.

5. Which viral infection is common in dialysis patients?

Hepatitis C virus is common in dialysis patients.

6. Which ELISA kit is used for the detection Hepatitis C virus?

Third generation ELISA kit is most commonly used for the detection which employ antigen from core, NS3, NS4 and NS5 to detect antigens from anti HCV antibodies.

#### **7.4. HIV Rapid card test**

The Test is a rapid, sensitive and accurate immunoassay for the differential detection of HIV-1 & HIV-2 antibodies (IgG) in human serum or plasma using HIV-1 & HIV-2 antigens immobilized on an immunofiltration membrane.

The test is a screening test for anti-HIV-1 & anti-HIV-2. 2.

##### **Principle**

HIV antigens are immobilized on a porous immunofiltration membrane.

Sample and reagents pass through the membrane and are absorbed into the underlying absorbent.

As the patient's sample passes through the membrane, HIV antibodies, if present, bind to the immobilized antigens.

Conjugate binds to the Fc portion of the HIV antibodies to give distinct pinkish purple DOT(s) against a white background.

##### **Method**

It is a rapid visual test for the Qualitative and differential Detection of Antibodies to HIV-1 & HIV- 2.

Add 3 drops of Buffer Solution to the centre of the device

Hold the dropper vertically and add 1 drop of patient's sample (serum or plasma) using the

sample dropper provided (use a separate sample dropper for each specimen to be tested).

Add 5 drops of buffer Solution.

Add 2 drops of liquid conjugate directly from the conjugate vial.

Add 5 drops of Buffer Solution and read results. Read results immediately and discard the device considering it to be potentially infectious.

To ensure assay validity, a procedural control is incorporated in the device control and labelled "Control".

If the control bars does not show any colour development by assay completion.

The test is invalid which could be because of following reasons:

Improper storage at temperature other than the recommended temperature.

Wrong Procedure like not adding serum, conjugate or substrate.

Long atmospheric exposure of the test device after opening the pouch.

This test procedure is same for Hepatitis A and Hepatitis C.

### **7.5. Hepatitis B rapid card test**

It is a rapid, sensitive and accurate one step immunoassay for the qualitative detection of Hepatitis B Surface Antigen (HBsAg) in human serum or plasma.

The assay is intended to be used as an aid in the recognition and diagnosis of acute infections and chronic infectious carriers of the Hepatitis B Virus (HBV)

It is a one-step immunoassay based on the antigen capture, or "sandwich".

#### **Principle:**

The method uses monoclonal antibodies conjugated to colloidal gold and polyclonal antibodies immobilized on a nitrocellulose strip in a thin line.

The test sample is introduced to and flows laterally through an absorbent pad where it mixes with the signal reagent.

If the sample contains HBsAg, the colloidal gold-antibody conjugate binds to the antigen, forming an antigen-antibody-colloidal gold complex. The complex then migrates through the nitrocellulose strip by capillary action.

When the complex meets the line of immobilized antibody (Test line) "T", the complex is trapped.

forming an antibody-antigen-antibody colloidal gold complex. This forms a pink band indicating the sample is reactive for HBsAg.

Assay procedure

Add 2 drops (70 µl) of human serum/plasma specimen into the sample well using the dropper provided. Allow reaction to occur during the next 20 minutes. Read results at 20 minutes.

Discard the card immediately after reading result at 20 minutes, considering it to be potentially infectious.

## 7.6. Hepatitis C rapid card test

It is a rapid, sensitive and accurate one step immunoassay for the qualitative detection for the detection of antibodies to Hepatitis C Virus in human serum or plasma.

Hepatitis C Virus was the main aetiological agent of non-A, non-B hepatitis (NANBH) accounting for greater than 90% of post-transfusion hepatitis cases. It is considered to be the major cause of acute chronic hepatitis, liver cirrhosis and hepatocellular carcinoma throughout the world. It is therefore necessary to correctly diagnose Hepatitis C infection.

### **Principle:**

Modified HCV antigens representing the immunodominant regions of HCV antigen. They are for the putative core (structural), protease/helicase NS3 (non-structural), NS4 (nonstructural) and replicase NS5 (non-structural) regions of the virus in the form of two test dots "T1" & "T2" and are

immobilized on a porous immunofiltration membrane. Sample and the reagents pass through the membrane and are absorbed into the underlying absorbent pad.

As the patient's sample passes through the membrane, HCV antibodies if present in serum/plasma, bind to the immobilized antigens. In the subsequent washing step, unbound serum/plasma proteins are removed.

In the next step, the protein-A conjugate is added which binds to the Fc portion of the HCV antibodies to give distinct pinkish purple dot against a white background at the test region ("T1" &/or "T2").

### **Assay procedure:**

Add 3 drops of buffer solution to the centre of the device.

Hold the dropper vertically downwards and add 1 drop of patient's sample (50 µl serum or plasma) using the sample dropper provided.

Add 5 drops of buffer Solution.

Add 2 drops of Protein- A Conjugate.

Add 5 drops of buffer Solution.

Read result immediately and discard the device immediately considering it to be potentially infectious

## **Questions**

1. What are the advantages of rapid test?

The advantage of rapid test is that the results can be interpreted immediately by visual examination.

2. How do you do HIV, Hepatitis B, Hepatitis C rapid card test?

Answer is explained in the procedure above.

3. What is the principle of HIV, Hepatitis B and Hepatitis C rapid card test?

Answer explained in the principle above.

## **8. Post-analytical stage**

Reporting and verifying of microbiological test results.

Taking appropriate action when a result has serious implications for a patient or public health, while interpreting test results correctly. The terminology and format used in reporting microscopical preparations culture, and antimicrobial sensitivity tests should be standardized and agreed between clinicians, lab personnel. Before leaving the lab, all reports must be checked for correctness and clarity and signed by the person in charge of the department.

Whenever possible the regional public health laboratory should organize an external quality assessment (EQA). The main objective of an EQA scheme is to confirm that a laboratory's SOPs.

(Standard operating procedure) and internal QC procedures are working satisfactorily. EQAs help to identify errors, improve the quality of work, stimulate staff motivation and assure users of the service that the lab is performing to the standard required to produce desirable results.

## **9. Health-care associated Infection**

## 9.1 Health-care associated Infection

Clinical microbiologists play a very important role in prevention of health care associated infections. Nosocomial infection is health care associated acquired infection. This is the infection the person acquires 48 hours after he/she is admitted in the hospital. As a clinical microbiologist you should have knowledge about MRSA (Methicillin Resistant *Staphylococcus aureus*), MDR (Multi Drug Resistant) and VRE (Vancomycin Resistant *Enterococci*) etc.

You should also have knowledge about CAUTI (Catheter associated urinary tract infection), CRABSI (central line associated blood stream infection), PLABSI (peripheral line associated blood stream infection), SSI (surgical site infection), VAP (ventilator associated pneumonia). The microbiologist should go for regular rounds to all the wards, OPDs (outpatient department) and theatres at least once a week along with an infection control nurse. Frequent visits may be required when there is an impending infection.

When you go for rounds you need to monitor for biomedical waste segregation, hand hygiene surveillance, labelling of 1% hypochlorite (its effective only for 24 hrs), hand sanitizers, normal saline bottles, xylocaine vials (validity for 30 days). You need to check sterile bins too. Look for the steritape if the indicator colour has changed. Ideally the bins are sterile only for a week. If it has not been used by then, it should be sent for sterilization again. You should have knowledge about CSSD (central sterile supply department), how items are collected there, packed, autoclaved and sent to respective OPDs, operation theatres etc.

It is the duty of Clinical Microbiologist to take necessary action to prevent nosocomial infection. In addition should also check for segregation of biomedical waste in the hospital which is explained in the respective sections.

## 9.2 Operation theatre – surveillance

There are certain criterias to be followed in operation theatres.

1. Only people absolutely needed for an assigned work should be present.
2. People present in theatre should make minimal movements and curtail unnecessary movements in and out of theatres, which will greatly reduce bacterial count.

Air borne contamination is usually affected by type of surgery, quality of air which in fact depends on rate of air exchange. All the persons including the least cadre of employers are partners in infection control and should be aware to comply with infection control regulations.

Prompt disposal of theatre waste out of the theatre is of top priority. The environments in the operation theatre are dynamic and subject to continuous change. Good infrastructures do not mean a safe

environment as humans make a greater difference in making the environment unsafe. Microbiologists should be aware of organisms, sites and populations as surveillance cultures should be chosen carefully to allow meaningful interpretation of results. Microbiologists should be familiar with the clinical techniques as those normally used for culturing clinical specimens may not yield correct results when applied to environmental specimens. Bacterial counts in the operation theatres are influenced by the number of individuals present, ventilation and air flow. All these should be considered when the results are interpreted.

Air handling filters need to be cleaned every 30 days of a month.

Factors influencing OT contamination

a. Type of surgery

b. Quality of air provided and rate of air exchange

c. Temperature / Pressure (should be positive pressure inside OT) / humidity

d. Movement of operating room personell

e. Level of compliance

### 9.2.1. Indication for air sampling

Generally regular surveillance is not required. There are two principle means of monitoring the microbiological parameters of the air: passive and active sampling. Testing for *Clostridium* spores are indicated if there is any reconstruction of the theatres or any modification done in the theatre. Use only vacuum cleaners for cleaning the floors.

The operation theatres should be fumigated regularly (weekly once) and should send culture plates (settleplate method) after fumigation. Fumigation is usually done with quaternary compounds. Usually 4th generation quaternary ammonium compounds are used. (Note: follow the instructions as guided by infection control committee in your hospital)

Earlier formalin was used for fumigation, now a days it is not recommended as it is toxic and carcinogenic.

Generally blood agar, SDA plates. are used for settle plate method. The plates are kept in 4 corners and also in the centre. **The plates should be kept for 1hr and should be 1metre from the wall, 1 metre from the floor on the OT table. (1,1,1 method).**

Bacteria settles down on to the culture plate, following which the plates should be incubated for 48 hours before you give the report. Results are interpreted as number of CFU per unit are per the duration of sampling time. (i.e. CFU/m<sup>2</sup>.hour).

The colony count should be less than 5. But if there is presence of one colony of pathogenic microorganism (either *Staphylococcus aureus*, *Pseudomonas aeruginosa*) it should be reported as unsatisfactory.

And also one fungal colony if present should be considered as unsatisfactory. The infection control team should inform the concerned person about the reports and take necessary action.

In active monitoring, a microbiological air sampler is used which physically draws a known volume of air through or over a particle collection device. Active monitoring is applicable when the concentration of microorganisms is not high such as in operating theatres.

### 9.3 CSSD (Central sterile supply department) – surveillance

We should not miss visiting CSSD (Central Sterile Supply Department) which is the back bone of the hospital. You need to go regularly and check the sterilization of surgical materials. Usually there is a uniflo working environment in CSSD. Need to look for the steritape in the bin and also look for the labelling of the strip. Ideally the bins used for dressing once sterilized should be used for a week. If it is not used by then, they should be sent for autoclave again. Similarly all surgical instruments should be sterilized as per the guidelines made by CSSD. The autoclave working condition should be checked weekly once by using biological indicator. The indicator should be kept in the centre of the autoclave machine and should be sent for culture to check if the spores were destroyed. The spore used is  $10^5$  *Bacillus stearo thermophilus*.

### 9.4 Water Surveillance

Hospital water supply may serve as a reservoir of health care associated water borne pathogens. Water can get contaminated with microorganisms present in hospital environment or if the sewage treatment plant is not maintained properly also there are chances of water contamination. The surveillance should be done for both drinking and washing water on a regular basis.

#### Principle

Water to be tested is diluted serially and inoculated in MacConkey purple broth, coliforms if present in water utilize the lactose present in the medium to produce acid and gas. The presence of acid is indicated by color change of the medium and the presence of gas is detected by the gas bubbles collected in Durham's tube present in the medium.

The number of total coliforms is determined by counting the number of tubes giving positive reaction.

(i.e. both color change and gas production) and comparing the pattern of positive results (the number of tubes showing growth at each dilution) with standard statistical tables (McCrady) to determine the MPN of coliform count present per 100ml of water.

**Labeling.** The container should be labelled with details of the tap locations, date and time of sampling.

**Transport:** The bottles after labelling should be transported to the lab and processed as quickly as possible atleast within 6 hours. If there is a delay, the samples can be refrigerated and processed within 24 hours.

**Procedure:**The container used for collection should be a screw capped borosilicate glass or plastic bottles of 500mL holding capacity, that has been cleansed, and rinsed with deionized or distilled water. When you are collecting water for testing allow the water to run for some time and then collect.

Do not touch the outer surface of the tap. At least 150-200ml of water should be collected. Do not collect from the tank. Inoculate 50ml water to one tube having 50ml of double strength medium, 10ml water added to each of 5 tubes containing 10ml double strength medium. 1ml of water added to each of 5 tubes containing 10ml of single strength medium. The indicator used is bromocresol purple.

The inoculated tubes are incubated at 37deg C for 48 hour. Positive change is indicated by color change in the medium from purple to yellow (due to lactose fermentation)

Gas gets collected in durham's tube.

After inoculating for 48 hours, see the colour changes in all tubes.

Positive test is indicated by a color change in the medium from purple to yellow, due to lactose fermentation, turbidity for the medium and gas collected in the Durham's tube.

The number of tubes giving the positive reaction is compared to a standard statistical chart ( Mccrady's chart for analysis). We are calculating the most probable number (MPN) of coliform organisms detected in 100ml of water.

For example if the tested samples shows 0-0-1, the sample is satisfactory. If the counts are like 1-4-1, the sample is unsatisfactory. After that you need to streak the water on Macconkey and blood agar and isolate the organism by doing biochemical reactions. The method is known as Ejeckman test.

Antibiotic sensitivity is not required as it is only water analysis. If you identify *E.coli* it is indicative of recent faecal contamination of water. The other organisms that can be detected are *Enterococcus spp.* (indicative of remote contamination of water), *Pseudomonas aeruginosa*, which plays an important role in hospital supply of water. When there is colony count of >10 per 100ml sufficient corrective measures to be taken.

You need to know about pour plate and spread plate method of water analysis.

**Pour plate method:** Water sample is added to molten agar and poured into petridishes and incubated at different temperatures. The result is expressed as number of colony forming bacteria per ml of water.

**Spread plate method:** The spread plate method involves spreading 0.1ml of the sample over a pre-made agar plate and allowing it to incubate. This method gives more three-dimensional colonies than the pour

plate method. The pour plate or spread plate methods are typically used for treated water and drinking water.

**9.5. Biomedical waste segregation:** Waste management in healthcare facility (HCF) includes-

1. Bio medical waste which includes infectious waste, radioactive waste, electronic waste.

2. General waste

Bio medical wastes are defined as wastes that are generated during the laboratory diagnosis, treatment or immunization of human beings or animals. These infectious wastes can affect health care workers by transmission through ingestion, inhalation or through direct skin- to skin contact and can result in various types of infections such as gastrointestinal, respiratory and skin infections.

Biomedical waste includes

(i) Infectious waste (10%)

(ii) Chemical or radioactive waste (5%)

Inappropriate and inefficient disposal of BMW can lead to infectious hazards, malignancies, malformation, and environmental (air, land and water) pollution.

Hazards associated with BMW

Hazards from radioactive waste: They are produced as a result of procedures performed by radiology and nuclear medicine. Hazards from cytotoxic waste: Used in the treatment of cancers and autoimmune disorders can lead to teratogenic or carcinogenic effects.

The new BMW guideline with an amendment in 2018 has been implemented with an easy of segregation, transportation and disposal of methods to decrease environment.

Steps of Bio medical waste management

Step 1- Waste segregation

Step 2- Pre-treat laboratory and highly infectious waste

Step 3- Collection and storage of segregated waste in color coded bags/containers/bins

Step 4- Intra- mural transportation from generation site to central storage area

Step 5- Storage

Step 6- Treatment (within 48 hours generation)

Step 7- Disposal.

**Waste segregation**—This is the most crucial step in biomedical waste management. Segregation should be done by using containers of 4 different colours.

**Yellow bag**—for infectious non plastic waste(Infectious non plastic waste which includes surgical gauzes, pads, animal tissues, human anatomical waste)

**Red bag**—for infectious plastic waste(Infectious plastic wastes which includes catheter bags, IV sets, Syringes).

**White or translucent sharp container** (puncture-proof box)—for sharp metal

**Blue container** --- for broken glass items and metal implants.

All these bags should be labelled, and the bags are non- chlorinated bags with a thickness of  $\geq 50\mu\text{m}$ . All thebags used for waste collection need to be sealed once they are full to 3/4th of their capacity.

Color coding	Waste category	Treatment options
Yellow	Infectious non plastic waste	Incineration/deep burial
Red	Infectious plastic waste	Autoclaving/microwaving/chemical treatment
Blue	Ampuoles, vials	Microwaving
White	Needles	Puncture proof container.
Green	General waste	Hand over to corporation

Onsite transport should take place during non - busy hours, whenever possible.

Dedicate trolley: General and hazardous waste should always be transported by separate trolley, painted with appropriate colors (black, yellow, and red etc..) and should have biohazard symbol.

HCWs handling BMW during transport or in the storage area should wear appropriate personal protective equipment (PPEs) such as heavy-duty gloves, facemasks, gowns and gumboots.

Trolley should be washed, disinfected and adequately dried up after each transportation cycle.

**Storage time:** Storage time for infectious waste (e.g. the time gap between generation and treatment) should not exceed more that 48 hours.

According to BMW rule 2016, India has recommended all segregated waste should be sent for final disposal within 48 hrs of collection.

Treatment and Disposal methods Various methods of disposal include

Incineration

Autoclave

Hydroclaving

Chemical disinfection

1. Effluent treatment plant
2. Microwaving
3. Mechanical process which include shredder, grinding and mixing
4. Deep burial
5. Sharp pit
6. Plasmapyrolysis

## **9.6. Spillage management**

Spillage of blood, body fluids, or chemicals in hospitals can occur at anytime either as a result of broken or faulty equipment or human error.

These spills pose a threat of infection (e.g. HIV, Hepatitis B and Hepatitis C) to the staffs, visitors and patients. So people who are handling should have knowledge about management.

Spillage of blood and body fluid can largely be prevented by adherence to the standard precaution while handling the blood and body fluid and through proper planning of collection and transportation.

In case if spillage comes in contact with skin, mucous membrane eyes or open wound, the first step is to irrigate continuously and to follow appropriate first aid measures. Do not rub your eyes and consult the ophthalmologist.

### **Management of spills**

Think all samples are infected

Keep a no entry sign board

Wear PPE ( personal protective equipment)

When sharps are involved, they can be picked up by using forceps and then discarded into a sharp box.

Disinfection: The spill area should be covered with freshly prepared 1% hypochlorite solution.

Put an absorbent material over that area. Wait for 20min

Needle pricks from sharps can lead to transmission of blood borne viruses ( hepatitis B,C and HIV).

Collect the materials and then discard in yellow bin

Wipe the area again with soap and water and then allowed to dry.

### **Spill kit**

This must be readily available in high-risk areas like laboratory, wards, sample collection rooms etc. The kit should contain the following

PPEs Gloves, apron, mask, shoe cover, eye protector

Absorbent materials like newspaper or blotting paper

Waste collection bag

Disposable forceps

Scoop and scraper or cardboard pieces

Sign for no entry

Buckets and mops of spills should be different from the regular mops and buckets should be cleaned separately

### **9.7. Needle stick injury**

Needle stick injury is very common in health care workers. In addition to needle injuries, injuries can be caused by other sharp objects (e.g. glass vials, surgical blades, forceps)

The risk for needle injury takes place in following circumstances

1. Reuse of syringes

2. Recapping needles

3. Overuse of injections

4. Unsafe sharp waste management practices which include inappropriate segregation of sharp waste.

5. The risk of infection following exposure depends on the following factors

Type of needle—hollow bore needle has a higher risk than solid needle

Depth of injury--- higher is the depth, more is the risk

Volume of blood involved in the exposure, viral load present in the blood/body fluid at the time of exposure, delay in start of appropriate.

PEP for HIV and Hepatitis B.

Most of the time Needle stick injury gets under reported.

Safety precautions always required to prevent injury.

Practice safe hand washing before and after the procedures, use of special containers for sharp disposal, use of needle cutter/destroyers, use of forceps instead of fingers for guiding sutures. HCWs must be immunized against Hepatitis B and must be checked for anti-HBs antibody titer ( $>10\text{mIU/mL}$ )

If there is an accidental prick how do you manage?

Post exposure prophylaxis refers to the comprehensive management instituted to minimize the risk of infection, following potential exposure to blood borne pathogens ( HIV,HBV,HCV).

If there is a prick and blood is oozing from the wound, don't attempt to squeeze the wound and show it under running water till the bleeding stops.

We need to report the infection control department and give a serum sample initially for testing of HIV, HBs Ag and HCV.

The serum has to be tested at an interval of 0,3 and 6months.

If the source is positive( from whom the prick has taken place) give them antiretroviral therapy for a period of 28 days

Questions?

- 1.What is health care associated infection?
- 2.Why do you think surveillance of CSSD and OT important?
- 3.How many plates are used for OT surveillance and how is it done?
- 4.How do you interpret water analysis using Mccrady's chart?
- 5.How do you manage spills in hospital?
- 6.How do you manage if there is accidental needle prick?
- 7.What are the various treatment and disposal of biomedical waste?
- 8.How do you manage biomedical waste in your hospital?

# 10.Format for culture seminar *S.aureus*

## Day 1.

Nutrient agar slope:

Confluent/discrete growth

Opaque with yellow pigmentation which was not diffusible ---- Hand lens required to view the colony

Odour (read odour from Koneman book)

### 1. Gram stain—was performed by Hucker's modification with suitable control

Positive control ATCC *S.aureus* 25923

Sample

Negative control ATCC *E.coli* 25922

Size of the smear 20x10mm

Thickness of the smear—The smear shouldnot be too thick or thin

It is assessed by you in such a way that you should be able to read the news paper in the back ground

Mention the method—Hucker's modification

**Interpretation--** Mention as control satisfactory.

The smear shows gram positive cocci arranged in clusters.

### 2. Catalase test

Two methods

Slide and tube catalase test

Tube catalase test 3% hydrogen peroxide

Positive control—ATCC *S.aureus* 25923

Test organism

Negative control—*Enterococcus faecalis* ATCC 29212 or *Streptococcus spp.*( mention references)

Interpretation: The given organism produced the enzyme catalase

### 3. Oxidase test you need to do modified oxidase test with 6% DMSO

If it is not there no need to do.

**Inference on day 1.** The given nutrient agar slope contains Gram positive cocci arranged in clusters, which is catalase positive belong to Staphylococcaceae family

#### Requirements for 2<sup>nd</sup> day

Liquid media : peptone water

Plating media

**For *S.aureus*—**

Nutrient agar-1 no (to observe colony morphology, pigmentation, and odour if any and for preliminary test)

5% sheep Blood agar -1no

Macconkey agar with out crystal violet –1no

Muller Hinton agar for antibiotic susceptibility testing-3 including control

Milk agar, Mannitol salt agar, Potassium tellurite agar, Phenolphthalein phosphate agar, 6.5%, NaCl, 10% NaCl agar, DNase agar plate ( number of plates 2 each including control)

Mcfarland standard (0.5)

CLSI interpretation chart

#### Enzyme detection

Coagulase test – Requirement's plasma 4 tubes, water bath.

Christensen's urease agar

#### Sugar detection (1%)

Mannitol, Trehalose, sucrose

Glucose phosphate broth for MR, VP ( reagent Barret's A and Barret's B)

#### Amino acid decarboxylation

Ornithine decarboxylate broth (*S.lugdunensis*)

OF medium to differentiate Micrococci and *S.aureus*

Apart from regular antibiotics for Gram positive cocci

Erythromycin(15ug),Clindamycin(2ug),cefoxitin(30ug),ciprofloxacin (5ug),Tetracycline(30ug),Gentamicin(10ug),

for Bacitracin(0.05units), lysostaphin,Novobiocin 5ug,furazolidine,cotrimoxazole(5ug), chloramphenicol(30ug)

## 2<sup>nd</sup> day interpretation

Nutrient agar—colony morphology opaque colony around 2-3mm in size,surface smooth,well defined edge non diffusible golden yellow colony pigment present.

Mac conkey agar without crystal violet—Lactose fermenting colonies present

Blood agar—beta hemolysis

Confirm your yesterday's finding from nutrient agar by doing gram stain,catalase, microoxidase test. **Note**

**you should not do catalase from blood agar plate as catalase present in blood agar will give false**

**positive result.If only blood agar alone is there then with a straight loop pick up only the colony and not the agar.**

*S.aureus*--- coagulase test – tube coagulase, slide coagulase both are positive.Urease—

hydrolysed

	<i>S.aureus</i>	<i>S.epidermidis</i>	<i>S.saprophyticus</i>
Mannitol	+ve	-ve	+ve
Trehalose	+ve	-ve	+ve
Sucrose	+ve	+ve	+ve
Novobiocin 5ug	sensitive	sensitive	resistant
coagulase	positive	negative	negative

OF—Oxidatively fermented in *S.aureus* whereas micrococci is oxidative.

Novobiocin resistant present in *S.saprophyticus* of CONS.

MHA look for zone size of cefoxitin disc.Surrogate marker for MRSA

**Use hand lens to describe the colony**

**Gram stain controls should be in same slide with markings**

**Label the catalase/oxidase tube with marker**

**Label all the necessary plates and tube**

## **Gram positive cocci (Enterococcus)**

### **Day 1.**

Nutrient agar slope (Confluent/discrete growth, opaque, non-pigmented) -- Hand lens required to view the colony

Odour (after you open the tube)

### **1. Gram stain—was performed by Hucker's modification with suitable control**

Positive control ATCC *S.aureus* 25923

Sample

Negative control ATCC *E.coli* 25922

Size of the smear 20x10mm

Thickness of the smear—The smear should not be too thick or thin

It is assessed by you in such a way that you should be able to read the newspaper in the background

Mention the method—Hucker's modification

**Interpretation--- Mention control satisfactory and your smear is gram positive cocci arranged in pairs.**

### **Catalase test**

Two methods

Slide and tube catalase test

Tube catalase test 3% hydrogen peroxide

Positive control—ATCC *S.aureus* 25923

Test organism

Negative control—Enterococcus spp( 29212) or Streptococcus spp.( mention references)

**Interpretation: The given organism does not produce the enzyme catalase**

### **3. Oxidase test you need to do modified oxidase test with 6% DMSO**

If it is not there no need to do.

**Inference on day 1.** The given nutrient agar slope contains Gram positive cocci arranged in pairs which is catalase negative belong to Streptococcaceae family.

## Requirements for 2<sup>nd</sup> day

Liquid media : peptone water

Nutrient agar-1 no

5% sheep Blood agar -1 no

Macconkey agar with and without crystal violet –1 no each.

Muller Hinton agar for antibiotic susceptibility testing-3 including control

Mcfarland standard (0.5)

Water bath – Heat tolerance test

Growth at 10 deg and 45 deg for enterococci.

Glucose phosphate broth ( MR , VP reagent)

Sugars( 1%)—Mannitol, Arabinose, pyruvate

Amino acid- arginine

Bile esculin

Antibiotics for Enterococcus—High level Gentamicin(120ug), Penicillin(10units), Ampicillin(10ug), Vancomycin(30ug),Ciplox.(5ug),Erythromycin(15ug),Tetracycline(30ug),Linezolid(30ug),chloramphenicol(30ug) (CLSI 2021).

## 2<sup>nd</sup> day

Confirm your yesterday's finding by doing gram stain,catalase, oxidase, hanging drop from Nutrient agar plate

Nutrient agar—colony morphology size,surface, consistency, edge ,pigment

Mac conkey agar— magenta colourcolonies,Lactose fermenting colonies

6.5% Nacl agar

Blood agar—non hemolytic

VP positive

Bile esculin hydrolysed.

Arginine deamination both for *E.faecalis* and *E.faecium* is positive.( arginine dihydrolysed)

Heat tolerance test positive for *E.faecalis* and *E.faecium*

Sugars	<i>E.faecalis</i>	<i>E.faecium</i>
Mannitol	Positive	Positive
Arabinose	negative	positive
Pyruvate	Positive	positive

**Use hand lens to describe the colony**

**Gram stain controls should be in same slide with markings**

**Label the catalase/oxidase tube with marker**

**Label all the necessary plates and tube**

### **Enterobacteriaceae**

PURE CULTURE- *Kleb. pneumoniae*

Pure culture on nutrient agar slope was provided for identification and complete phenotypic characterization of given organism

Nutrient slope - It showed medium sized colonies, smooth low convex surface, glistening and densely opaque. No odour or pigmentation was appreciated.

Preliminary test were performed:

Gram stain- was performed by Hucker's modification with suitable controls

Positive control – *Staphylococcus aureus* ATCC 25923 and

Negative control – *Escherichia coli* ATCC 25922

Controls were satisfactory and given organism was uniformly stained, is a Gram negative bacilli with parallel sides and rounded edges with clear halo around the bacilli.

Catalase Test: Was performed by tube method with freshly prepared 3% H<sub>2</sub>O<sub>2</sub> using suitable controls.

Positive control – *Staphylococcus aureus* ATCC 25923

Negative control – *Enterococcus faecalis* ATCC 29212

Controls were satisfactory. The given organism produced the enzyme catalase.

Oxidase test: Was performed by wet-film paper method with freshly prepared 1% oxidase reagent

with suitable control.

Positive control – *Pseudomonas aeruginosa* ATCC 27853

Negative control – *Escherichia coli* ATCC 25922

Controls were satisfactory. The given organism did not produce the enzyme oxidase.

Motility test:

Was performed by Hanging drop technique from nutrient broth culture.

The given organism shows non-motile.

Inference on day 1:

The given organism shows Grams negative bacilli arranged in short, non-motile positive for catalase and negative for Oxidase. The organism belongs to the Family Enterobacteriaceae.

Requirements for Day 2:

Culture plates:

Nutrient agar-1 (to observe colony morphology, pigmentation, and odour if any and for preliminary test)

MacConkey agar-1

5% Sheep Blood agar-1

Mueller Hinton agar- 2

Mcfarlands Standard (0.5)

ATCC *Escherichia coli* culture, CLSI interpretation chart

Preliminary Screening media:

Indole

Triple sugar iron agar

Mannitol – Motility test

Phenylalanine agar

Test for Enzymes:

Christensen`s urease agar 1

Nitrate reduction test

Test for citrate utilization as sole carbon and energy source

Simmon`s citrate agar

Test for breakdown products

Methyl red & Voges-Proskauer broth

MR reagent and VP reagent (A & B)

Test for 1% Sugar fermentation: Glucose, Sucrose, Lactose, Salicin, Adonitol, Inositol, Sorbitol, Arabinose, Raffinose, Rhamnose, Maltose, Xylose, Trehalose, Mannose (based on history/ preliminary reduce the sugar test)

Test for Oxidative-Fermentative:

OF-glucose- 6 tubes (Respective controls Oxidative- *Pseudomonas aeruginosa* ATCC 27853, fermentative- *Escherichia coli* ATCC 25922), paraffin oil

Test for Amino acid decarboxylase:

LOA Base,

Lysine, Ornithine, Arginine (2 tubes for each)

If salmonella or shigella suspected -ask for antisera for serotyping.

*Salmonella typhi* O antisera

*Salmonella typhi* H antisera

*Salmonella paratyphi* AH antisera

*Salmonella paratyphi* BH antisera

Antibiotic sensitivity disc for AST:

Ampicillin (AMP – 10 mcg), Cefoxitin (CX – 30 mcg), Ceftazidime (CAZ – 10 mcg),

Ceftazidime/Clavulanic acid (CAC – 30/20 mcg) - ESBL, Gentamicin (GEN 10 mcg), Amikacin (AK –

30 mcg), Piperacillin/Tazobactam (PIT – 100/10 mcg), Ciprofloxacin (CIP – 5 mcg), Co-Trimoxazole

(COT – 25 mcg), Imipenem (IPM – 10 mcg)

**DAY-2**

Culture Characteristics:

On nutrient agar - Colonies are 2-3 mm in diameter, white mucoid, convex, smooth surface, entire

edges, non - pigmentation is observed.

On MacConkey agar – Colonies are 2-3 mm in diameter, mucoid lactose-fermented, convex, round smooth surface and entire edges.

On 5% Sheep Blood agar - Colonies are 2-3 mm, non-haemolytic, mucoid greyish white, convex, smooth, entire edges.

Repeat the preliminary tests from nutrient agar plate- from sub cultured plate.

Grams stain: Gram negative bacilli arranged predominantly in short- Controls were satisfactory.

Catalase Test: Positive- Controls were satisfactory.

Oxidase test: Negative- Controls were satisfactory.

Nitrate reduction test: Reduced to nitrite.

Motility test: Non-motile

#### **Biochemical test:**

Indole – No cherry red color ring seen

Triple sugar iron agar – Acid slant/Acid butt with Gas, without H<sub>2</sub>S production

Mannitol - motility media - Mannitol is fermented and organism is non motile

Urease agar - Hydrolysed

Citrate agar - Utilized

Phenylalanine agar – Negative

#### **Test for Enzyme:**

Christenson urea agar - Urea is hydrolysed

Nitrate Broth - The org was inoculated overnight, first add reagent 1 and then reagent 2 is added. Nitrate is reduced to nitrite (red)

#### Test for Breakdown products:

MR& VP broth with MR reagent & VP reagents (A&B)

Methyl red: negative

Voges-Proskauer test is changed to red color- POSITIVE (Acetoin produced)

(Note: Usually it takes 48h for report)

Test for Oxidative-Fermentative metabolism (Hugh Leifson Test):

OF-glucose- 6 tubes (Respective controls Oxidative- *Pseudomonas aeruginosa* ATCC 27853, fermentative- *Escherichia coli* ATCC 25922) was satisfactory.

Oxidative- fermentative – Organism shows fermentative metabolism.

Test for Amino acid decarboxylase:

LOA Base - Org is viable (yellow)

Lysine - Decarboxylated

Ornithine - Not decarboxylated

Arginine – Not dihydrolysed

Test for 1% sugar Fermentation:

Glucose –Fermented with/with out gas

Sucrose – Fermented with/with out gas

Lactose - ...fermented with/with out gas

Maltose , Mannose

Sorbitol ,Salicin

Arabinose, Raffinose , Rhamnose , Adonitol , Xylose Trehalose , Inositol

Antibiotic sensitivity disc for AST

Disc Test Control Inference

### **Final conclusion:**

The given organism was - Gram negative short bacilli which is Non-motile, Catalase (+), Oxidase (-), with mucoid lactose – fermented colonies, which reduced nitrate, produced Acetoin, Utilized citrate, Hydrolyzed urea and fermented all sugars with gas.

FAMILY: Enterobacteriaceae

TRIBE: *Klebsiellae*

GENUS: *Klebsiella*

SPECIES: *pneumoniae*

SUB-SPECIES: *pneumoniae*

**Use hand lens to describe the colony**

**Gram stain controls should be in same slide with markings**

**Label the catalase/oxidase tube with marker**

**Label all the necessary plates and tube**

Serology

CRP, RPR, ASO, RA

Follow the test as instructed in kit insert

All these are passive agglutination test.

Interpret as positive or negative

If it is positive need to know the titre value.

It is a qualitative test. Semi quantitative test is done by dilution of the serum/

Blood widal

Need to do tube agglutination test

Take 0.4ml of serum and 3.6ml of saline in one tube.

You need to take 4 rows of 6 test tubes for S.Typhi H, S.Typhi O, S.Paratyphi AH, S.Paratyphi BH

Add 1ml of saline to all the tubes. To the first tube add 1ml of diluted serum. From the 1<sup>st</sup> tube take 1ml and add to the second tube. Keep diluting like this and from the last tube take 1ml and discard.

Add 1 drop (50ul) of antigen to all the tubes. Keep it in water bath for 1hr and then keep at room temperature or keep it in incubator.

Look for agglutination in all the tubes.

You need to check for the titre.

Interpretation

S.Typhi O titre > 1:80 titre

S.Typhi H titre > 1:80 titre

S.Typhi AH titre < 1:20 titre

S.Typhi BH titre < 1:20 titre

Interpretation: Could be enteric fever. Advise repeat sample after a week for a rise in titre.

STO	STH	SPAH	SPBH	Interpretation
+	-	--	-	Enteric fever
+	+	-	-	Typhoid fever
-	+	-	-	Typhoid fever
+	-	+	-	Paratyphoid A fever
-	-	+	-	Paratyphoid A fever
-	+	+	-	TA vaccination
+	+	+	-	Recent TA vaccination
-	-	-	+	Sal.Gr.B infection
-	+	+	+	Anamnestic reaction

Interpretation

+80 for one antigen only Could be due to ----- fever

+ 80 for both STO and any one H. Suggestive of----- fever

+160 for any one antigen only ----Suggestive of----- fever

+ 160 for both STO and any one H only---Diagnostic of----- fever

+320 for any one antigen only---Diagnostic of ----- fever

# 11. Miscellaneous questions

## Urine culture

1. What are the different causative agents responsible for urinary tract infections?

Gram-negative bacilli:

*Escherichia coli*: Most common

*Klebsiella pneumoniae*

*Proteus mirabilis*

*Pseudomonas aeruginosa*

*Acinetobacter species*

*Enterobacter species*

*Serratia species*

Parasites:

*Schistosoma haematobium*

*Trichomonas vaginalis*

Gram-positive cocci

*Staphylococcus saprophyticus*

*Staphylococcus aureus*

*Staphylococcus epidermidis*

*Enterococcus species*

Viruses:

Herpes simplex virus

Adenovirus

JC and BK virus

Cytomegalovirus

2. What is mid-stream urine?

Midstream is collection of urine after initial voiding. Collecting only the mid portion.

3. What is upper UTI and lower UTI?

Upper UTI involves infection of kidney and ureter.

Lower UTI involves infection of urethra and bladder.

4. Which specimen is the best for urine examination?

Mid-stream urine is the best specimen for urine examination.

5. How many pus cells are normally present in male and female urine specimen respectively?

The normal count of pus cells in male urine samples is around less than 4 cells / HPF, whereas for females, the normal pus cells count is around 5 to 7 cells / HPF.

6. What is the importance of RBC in microscopic examination?

RBCs in urine is not normal. If present it indicates inflammation or infection of tissues of the urinary tract.

7. What are different types of casts?

Hyaline cast, waxy cast, epithelial cell cast, RBC cast, WBC cast.

8. What is significant bacteriuria?

A count of  $>10^5$  colony forming units (CFU)/mL of urine is considered as significant. It indicates infection (referred to as 'significant bacteriuria')

## **Blood culture**

1. What are the causes of septicaemia?

Urinary tract infection, Kidney infection, burns, lung infection, Germs on equipment, abscessed tooth are responsible for septicaemia.

2. What are the causes of bacteraemia?

*S.aureus*, *E.coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, Group A *Streptococcus*, *Salmonella Spp* are some of the bacterial pathogens for bacteraemia.

3. How do you collect blood specimen from patient?

Usually brachial vein is used and paired blood cultures are sent. The site needs to be cleaned with povidone iodine cleaning from centre to periphery and once cleaned do not touch the centre. Again clean with surgical spirit before collecting the sample.

4. Why do we need to collect 2 sets in blood cultures?

This is to avoid contamination as it is unlikely to get in 2 sets. The collection of larger volumes of blood increases the likelihood that microorganisms will be detected if present and also will neutralise the complement present in blood.

5. What are the different liquid media used for blood culture?

Yellow top – paediatric aerobic (0.5–4 mL blood)

Green top – adult aerobic (5–10 mL blood)

Orange top – anaerobic (5–10 mL blood)

Black top – mycobacteria (5–10 mL blood)

Silver top – mycoplasma (3–5 mL blood)

6. What is the importance of quantity of blood collected and the quantity of liquid culture medium?

Usually sample is collected in 1:10 dilution eg. 10ml for 50ml broth bottle to reduce the antimicrobial constituents to a sub effective level.

7. Why blood specimen is diluted in liquid medium?

The dilution is done to reduce the antimicrobial constituents to a sub effective level.

8. Name some automated blood culture systems.

Bacteck, BacT alert, Versa trek

9. Describe the functioning of automated blood culture systems used in your lab?

When microorganisms are present in culture vials, they metabolize nutrients in the culture medium, releasing carbon dioxide into the medium. A dye in the sensor at the bottom of the vial reacts with CO<sub>2</sub>. This modulates the amount of light that is absorbed by a fluorescent material in the sensor. A photodetector at each station measures the level of fluorescence, which corresponds to the amount of CO<sub>2</sub> released by organisms. Then the measurement is interpreted by the system according to pre-programmed positivity parameters. Positive cultures are immediately flagged by an indicator light on the front of the instrument, an audible alarm, and are displayed on the LCD display.



