Semester - II

I9MBP212 ADVANCED PRACTICAL–IV 4H – 2C

Instruction Hours / week: L: 4 T: 0 P: 4 **Marks:** Internal: **40** External: **60** Total: **100**

End Semester Exam: 9 Hours

- **COURSE OBJECTIVES**

A student undertaking this course will learn

- To acquire practical knowledge in numerous diagnostic tests and procedures used in the microbiology laboratory.
- To understand the importance of diagnostic procedures and gain skills related to the laboratory experiments.

COURSE OUTCOME

- This course provides the current medical aspects on the clinical diagnosis of infection providing the combined treatment of bacteriology and virology.

EXPERIMENTS

1. Laboratory diagnosis of pyogenic infections – tuberculosis – enteric fever –diarrhea – UTI – anaerobic infections
2. Isolation and identification of *Candida albicans*
3. Antibiotic sensitivity test disc preparation
4. Antibiotic sensitivity test – Kirby - Bauer, Stroke's method
5. MIC determination by Broth dilution technique, filter paper disc assay
6. Wet mount preparation of parasites- Saline, iodine
7. Identification of parasites-formal ether concentration, floatation methods
8. Morphological examination of fungi in tissues
9. Cultivation of viruses-Egg inoculation
10. Isolation of coli phage from sewage using membrane filter technique.
11. Examination of plant diseases: Wilt of potato, Citrus canker, Rice dwarf virus

SUGGESTED READINGS

1. Arora, B., and Arora, D.R., (2007). Practical Microbiology, (1sted.). CBS Publishers and Distributors,Bangalore.
2. Cappucino, G.J., and Sherman, N., (2001. Microbiology A Laboratory Manual. (6thed.). Benjamin Cummings, New York.
3. Baron, E.O., and Finegold, S., (1990). Bailey and Scott's Diagnostic Microbiology. (8thed.). C V Mosby Company, StLouis.
4. Gaud, R.S., and Gupta, G.D., (1999). Practical Microbiology. (1sted.). NiraliPrakashan,Pune.
5. Mukherjee, K.L. (2005). Medical Laboratory Technology, Vol. 3, Tata McGraw-Hill Publishing Company Ltd, NewDelhi.
6. Reddy, S.M., and Reddy, S.R., (2004). Microbiology A Laboratory Manual. (3rded.). Sri Padmavathi Publication, Hyderabad.
7. Sundararaj, T. (2005). Microbiology laboratory manual.AswathySundararaj Publishers. Chennai.
8. Vandepilte, J., Verhaegan, J., Engbaek, K., Rohner, P., Prot, P., and Heuck, C.C., (2004). Basic Laboratory Procedures in Clinical Bacteriology. (2nded.). A.I.T.B.S Publishers and Distributors, Delhi

ADVANCED PRACTICAL IV

SYLLABUS

1. **Laboratory diagnosis of**:
   a) Pyogenic infections
   b) Tuberculosis
   c) Enteric fever
   d) Diarrhea
   e) UTI
   f) Anaerobic infections
2. **Isolation and identification of *Candida albicans***
3. **Antibiotic sensitivity test disc preparation**
4. **Antibiotic sensitivity test:**
   a) Kirby – Bauer Method
   b) Stroke's method
5. **Minimum Inhibitory Concentration determination by**
   a) Broth dilution technique
   b) Filter paper disc assay
6. **Wet mount preparation of parasites:**
   a) Saline Wet mount
   b) Iodine Wet mount
7. **Identification of parasites:**
   a) Formal Ether Concentration
   b) Floatation Methods
8. **Morphological examination of fungi in tissues**
9. **Cultivation of viruses-Egg inoculation**
10. **Isolation of coli phage from sewage using membrane filter technique.**
11. **Examination of plant diseases:**
    - Wilt of potato
    - Citrus canker
    - Rice dwarf virus

EXPERIMENT NO: 1a

LABORATORY DIAGNOSIS OF PYOGENIC INFECTIONS

SAMPLE: **PUS**

Pus is an inflammatory virulent which contains leucocytes (Mostly neutrophiles) deoxy ribonuclease proteins and lysosomal incense from the decaying leucocyte, Hence pus producing microorganisms are called pyogenic microorganism or pyogens.

Wound is an abnormal break in the skin or other tissue which allows blood to escape. Wounds are of two types. They are open wound and closed wound, open wound allows blood to escape from the body were the skin is broken. All open wounds are contaminated with germs which enter from air, fingers and other part of the body. Any wounds has not begin heal property after 48 hours it may be infective. Infection may further spread and cause dangerous illness to the human being. Closed wound are due to the injury to the internal tissue where the out flow of the pus do not occur. This pus filled cavity is called abscess. *Staphylococcus aureus* mostly isolated from the skin wounds. *Pseudomonas aeroginosa* is associated with infected burns and hospital occurred infections. *Eshcherichia coli*, Proteus species are associated with abdominal abscess. *Clostridium perfringens* are closely found in deep wounds.

ORGANISMS INVOLVED IN PYOGENIC INFECTIONS:

Gram Negative Bacteria: *Pseudomonas aeroginosa, Proteus* sp., *E. coli, Klebsiella* sp.

Gram Positive Bacteria: *Staphylococcus* sp, *Streptococcus* sp, *Clostridium* sp, *Enterococcus*, sp

Fungus: *Candida* sp, *Histoplasmas* sp, *Cryptococcus neoformans*

MATERIALS REQUIRED:

- Sterile leak from proof container
- Sterile cotton swab
- **Media**: Ames transport medium, Blood agar, Neomycin Blood agar Robertson's cooked meat medium, Macconkey agar.
- Antibiotic discs

SPECIMEN COLLECTION:

Pus is collected from wound after initial disinfection of the skin, using spirit cotton to prevent commensals contamination. Pus can be collected from wound and abscesses. Pus from wounds is

collected using a sterile swab by spontaneous rupture, collected pus materials is transport into sterile leak proof container containing Amies transport media.

SPECIMEN PROCESSING:

MACROSCOPIC AND MICROSCOPIC EXAMINATION:

Colour, consistency and nature of the specimen examined before starting microscopy. Microscopy, direct examination using gram staining was done to observe morphology of the pyogen.

CULTURING:

- Inoculate the specimen into Blood agar, Neomycin Blood agar, Macconkey agar, Robertson's cooked meat medium.
- Incubate Blood Agar, Macconkey Agar aerobically at 35 to 37°c overnight.
- Incubate neomycin blood agar and blood agar plate anaerobically at 35 to 37°c for 48 hours.
- Incubate Robertson's cooked meat media at 35 to 37°c for 72 hours.
- Observe colony morphology and different medium and record the results.
- Subjected the isolate colonies to biochemical test for species identification.

RESULT:

The given pus sample was processed and positive agent was identified as _____. The results were tabulated below:

DIAGNOSIS OF PYOGENIC INFECTION

STAINING TECHNIQUE	**OBSERVATION**
Gram's Staining	
Motility Test	
BIOCHEMICAL CHARACTERISTICS	**OBSERVATION**
Indole Production	
Methyl Red	
Voges Proskauer	
Citrate Utilisation	
Triple Sugar Iron test	
Catalase Test	
Oxidase Test	
CULTURAL CHARACTERISTICS	**OBSERVATION**
Nutrient Agar	
Mac Conkey Agar	
Blood Agar	

ANTIBIOTIC SENSITIVITY TESTING:

The organisms present in the pus sample which is sensitive to (antibiotics) hence there are these antibiotics can be recommended for treating this pyogenic infection.

ANTIBIOTIC SUSCEPTIBILITY TEST

ANTIBIOTIC DISC WITH UNITS	**ZONE OF INHIBITION IN MILLIMETER**	**SUSCEPTABILITY**

EXPERIMENT NO: 1b

LABORATORY DIAGNOSIS OF TUBERCULOSIS

SAMPLE: SPUTUM

Sputum is the mixture of bronchial secretion and inflammatory exudates. In some bacterial infection there will be difficulty in sputum secretion. The sputum will be purulent with yellow or greenish colour which is usually a mixture of pathogenic microbes and commensals.

Lower respiratory tract infection involves lungs and bronchia, which are sterile organs. Any organism that is capable to bypass the host defence enter into the system, multiply and capable of causing diseases.

POSSIBLE PATHOGENS:

Mycobacterium tuberculosis, other organism in sputum are *Staphylococcus aureus, Streptococcus pnumoniae, Klebsiella pneumoniae,* etc.*.,*

MATERIALS REQUIRED:

- Disposable wide mouth sterile plastic container
- **Media**: Lowenstein Jensen media,
- **Reagents**: NaOH, Acid fast staining reagent.

TRANSPORTATION:

Sputum is transported or stored by adding cetylpyridinumchloride– sodium chloride (CPC-NaCl). It digest the sputum and prevents the over growth of other pathogens.

SPECIMEN PROCESSING:

Collection of specimen- Collect the early morning sputum or contains pooled over night secretion with concentrated bacteria. A sterile wide mouth container with a right screw cap bottle can be used to collect the sputum sample.

PROCESSING:

Most sputum is not homogenous. The sputum should be homogenized to remove the thick consistency.

ACID FAST STAINING:

A smear was made on the clean grease free glass slide and acid fast staining was performed. The smear was subjected under oil immersion objective lenses and viewed microscopically to observe the morphology of the pathogens.

CULTURING:

About 20 minutes before culturing decontaminate the specimen by mixing equal volumes of sputum and sodium hydroxide. Shake at intervals to homogenized the sputum.

Using a sterile premark Pasteur pippette inoculated 200 microlitre of sputum on a slope of acid Lowenstein Jensen medium and allows the specimen to run down the slope.

Slope turns yellow due to alkalinity of the specimen but it will became green again (acid in the medium neutralizes the NaOH).

Incubate the tube at 37°c in a rack placed at an angle of 45°c to ensure that the specimen is in contact with full length of the slope.

After one week placed the slope in an upright position and continue to incubate the culture for further 5 to 6 weeks, examine twice a week for growth.

RESULT:

The results were tabulated below

BARLETT GRADE SPUTUM

NO. OF NEUTROPHILS FIELD	GRADE
<10	
10-25	
>25	
Presence of mucous	
No. of epithelial cells per yield	
10-25	
>25	

EXPERIMENT NO: 1c

LABORATORY DIAGNOSIS OF ENTERIC FEVER

SAMPLE : BLOOD

Enteric fever is the term generally used for fever caused by *Salmonella* species namely S*almonella typhi, Salmonella paratyphi A and B* (typhoid paratyphoid fever). The presence of bacteria is blood called bacterimia. The term septicemia refers to a severe and often total infection of the blood in which bacteria multiply and release toxins in the blood streams. The symptoms of septicemia includes fever, chills and shock.

MATERIALS REQUIRED:

1. Sterile syringe and needle
2. **Reagents**: Gram staining reagents, 70% ethanol
3. **Media**: SS agar- Hektoen enteric agar, XLD agar, Bismuth sulfite agar , Rajhans medium, Macconkey agar, blood agar and chocolate agar

SPECIMEN PROCESSING:

Lab diagnosis of enteric fever includes in the following

- Microscopic method
- Culturing method
- Biochemical method
- Immunological method
- Antibiotic method
- Bacterial diagnosis of enteric fever consist of isolation of bacillus (*Salmonella species*)from the patients demonstration of the presence of antibodies in the patient serum

MICROSCOPIC METHOD

GRAM STAINING:

The blood sample is stained using gram staining method.

CULTURE METHOD:

The sample was inoculated in Macconkey agar, blood agar and chocolate agar, Hektoen enteric agar, XLD agar, SS agar act as a selective media for salmonella species. The sample were inoculated on the plate and incubated for 24hr at 37°c.

BLOOD CULTURE:

- About 5 to 10 ml of blood was collected by vein puncturing and inoculated in to a culture bottle containing 5 to 10 ml of 1% bile product broth.
- After overnight incubation at 37°c the bile broth was sub cultured on Macconkey agar plate and then incubated.
- The non-lactose fermentation colonies were taken for biochemical and motility test. The result were noted.

BIOCHEMICAL TEST AND ANTIBIOTIC SENSITIVITY TESTING:

Biochemical test for the colonies obtain from the above mentioned media were performed and antibiotic sensitivity test by Kirby bauer method.

RESULT:

The given blood agar sample were processed and causative agent are identified as _____. The microscopic and biochemical test are observed and tabulated below.

DIAGNOSIS OF ENTERIC FEVER

STAINING TECHNIQUE	OBSERVATION
Gram's Staining	
Motility Test	
BIOCHEMICAL CHARACTERISTICS	**OBSERVATION**
Indole Production	
Methyl Red	
Voges Proskauer	
Citrate Utilisation	
Triple Sugar Iron test	
Catalase Test	
Oxidase Test	
CULTURAL CHARACTERISTICS	**OBSERVATION**
Nutrient Agar	
Mac Conkey Agar	
Blood Agar	
Hektoen Enteric Agar	
XLD Agar	
Bismuth Sulfite Agar	
Rajhan's medium	

ANTIBIOTIC SENSITIVITY TESTING:

The organisms present in the sample sensitive to (antibiotics), hence this antibiotic is recommended for enteric fever.

ANTIBIOTIC SUSCEPTIBILITY TEST

ANTIBIOTIC DISC WITH UNITS	ZONE OF INHIBITION IN MILLIMETER	SUSCEPTABILITY

EXPERIMENT NO: 1d

LABORATORY DIAGNOSIS OF DIARRHOEA

SAMPLE: STOOL

Normal human intestine harbors more than 500 types of microbes. Among these microbes, some are considered as pathogens which may enter through food and water. Most of the intestinal disorders based on the toxin released.

Diarrhoea is a condition were intestinal discharge is expelled in a liquid state. Diarrhoea is caused by variety of microorganisms ranging from bacteria to virus. It leads to abdominal discomfort, pain with loss water and electrolyte which leads to dehydration.

POSSIBLE PATHOGEN

E.coli, Salmonella, Shigella, Enteritis, Vibrio Species, AcumbacterSpecies, etc…

COLLECTION AND TRANSPORT:

Stool is collected in a clean dry wide mouth container without urine contamination. The specimen containing mucus, pus is transferred into a leak proof container. When stool sample is not available or scanty a rectal swab is preferable. The sample should be transported using cary blair medium. The specimen should be transported as soon as possible. Do not refrigerate the stool sample certain species *Shigella* are susceptible to cooling and drying.

SPECIMEN PROCESSING

METHYLENE BLUE MOUNT:

On clean grease free glass slide places a drop of stool specimen and one drop of methylene blue. Cover slip was placed over it. This wet mount visualised under microscope for pus cells.

MOTILITY TEST:

Using hanging drop technique the stool sample suspended in saline was used to absorb the motility of the organism.

CULTURE METHOD:

The stool specimen was inoculated various medias like XLD Agar, Macconkey Agar, SS agar, This Sulphate citrate bio salt (TSCBS) incubated 24 hours at 37℃.

BIOCHEMICAL TEST:

Biochemical Test for the colonies obtained from the above mentioned media were performed and antibiotic sensitivity test was done.

RESULT

The given stool sample was processed and the causative agent was identified as_____.

Microscopic and biochemical Test were observed for and tabulated below:

DIAGNOSIS OF DIARRHOEA

STAINING TECHNIQUE	**OBSERVATION**
Gram's Staining	
Motility Test	
BIOCHEMICAL CHARACTERISTICS	**OBSERVATION**
Indole Production	
Methyl Red	
Voges Proskauer	
Citrate Utilisation	
Triple Sugar Iron test	
Catalase Test	
Oxidase Test	
CULTURAL CHARACTERISTICS	**OBSERVATION**
Nutrient Agar	
Mac Conkey Agar	
Blood Agar	
SS Agar	
TCBS Agar	

ANTIBIOTIC SENSITIVITY TESTING:

The organisms present in the pus sample which is sensitive to (antibiotics) hence there are these antibiotics can be recommended for treating diarrhoea.

ANTIBIOTIC SUSCEPTIBILITY TEST

ANTIBIOTIC DISC WITH UNITS	**ZONE OF INHIBITION IN MILLIMETER**	**SUSCEPTABILITY**

EXPERIMENT NO: 1e

LABORATORY DIAGNOSIS OF URINARY TRACT INFECTION

SAMPLE URINE.

Infection from any part from kidney till urethra is refered as urinary tract infection. Urinary infection may be normally acquired in hospitals following instrumentation like catheterization which lead to infection. The presence of bacteria on urine is called bacteremia. Significant bacteria are usually accompanied by pyuria (pus cell in urine). Infection of the bladder is called cystitis and infection of kidney is called pyelonephritis. *E.coli* is the commonest cause of urinary tract infection.

POSSIBLE PATHOGEN

Staphylococcous saprophytus, Pseudomonas aeruginosa, E.coli , Proteus sps, *Klebsiella* sps.

MATERIALS REQUIRED

Media: Blood agar, Macconkey agar, Cetrimide agar, Nutrient agar, Cystidine Lactose Electrolyte Deficient (CLED) agar.

SPECIMEN PROCESSING

SAMPLE COLLECTION

Mid stream urine, cathedral, suprapubic aspirations are the common urine collection methods. The urine is collected using sterile dry wide necked leak proof container. about 20ml of the sample should be collected., Urine is good medium for the growth of coli form and other urinary pathogens. Hence delayed in process will interfere in the result and provides error. Urine should be refrigerated at 4℃ if the delivery of the urine specimen to the laboratory more than 1hour than boric acid should be added.

MACROSCOPIC OBSERVATION

Observe the appearance of the specimen colour, consistency and nature of the specimen. Normally freshly passed urine is clear and pale yellow - yellow depending on their concentration. When left to stand cloudiness may develop due to the precipitation of urates in acid urine (or) phosphate and carbonates in alkaline urine. The presence of urates makes the urine to appear pink (or) orange colour.

MICROSCOPIC OBSERVATION

WET MOUNTING

Wet mounting procedure was performed to find out bacteria present in centrifuged urine sample.

GRAM STAINING

Gram staining was performed to observe the presence of bacteria in urine sample.

CULTURING

- Approximate number of bacteria per ml of urine can be estimated by usually calibrated loop technique (0.002 ml capacity or 500ml or 20×500 which is equal to 10000).
- Dilute the urine 10^{-5} and perform streak plate technique.
- Divide nutrient Agar plates into 6 portions and inoculate loop full of urine respective portion in single line.
- For selective isolation mix urine properly and inoculate a loop full of urine on blood Agar, Macconkey agar, Cetrimide agar, Cystidine Lactose Electrolyte Deficient (CLED) agar and SS agar.
- Incubate all plates at 37℃ for 24hrs observe colonies and interpret the result.

ESTIMATION OF BACTERIAL NUMBERS

Total numbers of colonies were calculated and total number of bacteria per ml of urine was estimated. 1000 colony-forming units (CFU)/mL and more than 10,000 CFU/mL, respectively, for clean-catch midstream urine specimens. The result interpreted is significant of urinary tract infection.

RESULT

The given Urine sample was processed and positive agent was identified as _____.

The results were tabulated below:

DIAGNOSIS OF URINARY TRACT INFECTION

STAINING TECHNIQUE	**OBSERVATION**
Gram's Staining	
Motility Test	
BIOCHEMICAL CHARACTERISTICS	**OBSERVATION**
Indole Production	
Methyl Red	
Voges Proskauer	

Citrate Utilisation	
Triple Sugar Iron test	
Catalase Test	
Oxidase Test	
CULTURAL CHARACTERISTICS	**OBSERVATION**
Nutrient Agar	
Mac Conkey Agar	
Blood Agar	
Cetrimide agar	
CLED Agar	

ANTIBIOTIC SENSITIVITY TESTING:

The organisms present in the urine sample which is sensitive to (antibiotics) hence there are these antibiotics can be recommended for treating this urinary tract infection.

ANTIBIOTIC SUSCEPTIBILITY TEST

ANTIBIOTIC DISC WITH UNITS	ZONE OF INHIBITION IN MILLIMETER	SUSCEPTABILITY

EXPERIMENT NO: 1f

LABORATORY DIAGNOSIS OF ANAEROBIC INFECTION

INTRODUCTION:

Anaerobes are bacteria which are capable of living on relative or total absence of oxygen. There are two types of anaerobes:

1) Spore forming or Clostridial anaerobes
2) Non-spore forming or non-clostridial anaerobes.

Anaerobic infections are usually endogenous and are caused by tissue invasion by bacteria normally resident on the respective body surface. Anaerobic bacteria are normally present on the skin, mouth, nasopharynx and upper respiratory track, intestines and vagina. Anaerobic infections generally occurs in patients with Trauma, Tissue necrosis, impaired circulation Hamatoma formation or the presence of foreign bodies. Diabetes, malnutrition, malignancy or prolong treatment with amino glycoside antibiotics may actually act as a predisposing factor. Anaerobic infections are typically polymicrobial. While the infection is usually localized general dissemination may occur by bacterimia.

There are some clinical features which suggest the presence of anaerobic infections.

- Pus produced by anaerobes is characteristically purulent with a pervasive, nauseating order.
- Pronounced cellulitis is a common feature of anaerobic wound infection.

ANAEROBES:

Clostridium tetani, Clostridial septicum, Peptococcus, Peptostreptococcus species etc.,

LABORATORY DIAGNOSIS:

COLLECTION OF SPECIMEN:

Proper collection of specimen avoiding contamination with normal Flora is absolutely essential.

TRANSPORTATION:

Transport of specimen is a critical factor, effecting the ultimate success of the anaerobic culture. Anaerobes are sensitive to oxygen, to protect extremely oxygen sensitive bacteria from lethal effect of oxygen from the time of collection until it is incubated.

Media: Stuart's transport media, Cary Blair media, Robertson's cooked meat media etc.,

MICROSCOPY:

Gram staining smear examination is important because the culture results should be compared with gram stained result. In every effort made to observe the organisms cell morphology.

CULTURING:

Specimen is inoculated on both selective and non-selective media to ensure the culture of all anaerobes present in the specimen. The commonly recommend media for primary culture of anaerobes are Blood Agar, Neomycin Blood Agar, Neomycin- Kanamycin Blood Agar , Phenyl Ethyl Alcohol Blood Agar, Kanamycin, Vancomycin, Blood Agar, Mc Intosh Field Agar and Gas Pack Jar.

TREATMENT:

Penicillin is effective against most anaerobes. Tetramycin and chloramphenicol shows good result against anaerobic infections.

EXPERIMENT NO: 2

ISOLATION AND IDENTIFICATION OF *Candida albicans*

INTRODUCTION:

Candida albicans is a normal flora found in the membrane of respiratory tract also demonstrated frequently in skin culture. *Candida albicans* can be isolated from cutaneous lesions. Sputum sample, vaginal swap, pus, etc of infected and immunocompromised individuals. Diseased caused by *Candida* species is generally termed as Candidiasis. These include

- Cutaneous candidiasis
- Vaginal candidiasis
- Intestinal candidiasis
- Broncho pulmonary candidiasis
- Septicemia
- Endocarditis
- Vulvo vanginitis
- Cutaneous lesions
- Meningitis
- Oral thrush
- Candida granuloma

The main pathogenic species involved are *Candida albicans, Candida trophicals, Candida stellatoidae, Candida pseudotrophicals, Candida crusie, Candida parapsilosos, Candida gulliermondii, Candida viswanathi.*

AIM

To identify and isolated the Candida species from the given sample

PRINCIPLE

Candida albicans is pathogenic yeast like fungus that is found both in culture and infected tissue. The surface growth consist of pseudomycelium which is composed of pseudohyphae on sabourdard dextrose agar they produce soft cream coloured colonies.

Candida albicans ferment glucose, maltose and produce acid and gas. It produces acid from sucrose and does not utilize lactose. Their carbohydrates fermentation together with colony

morphological characteristics differentiates *Candida albicans* from other species of *Candida*. Candida. Chromogenic agar is a selective and differential media for Candida species

This medium is especially used to differentiate the species of *Candida*.

CANDIDA CHROMOGENIC AGAR

COMPOSITION

Glucose	: 2g
Peptone	: 1g
Chloramphenicol	: 0.05g
Chromogenic mix	: 0.004g
Agar	: 1.5g
pH	: 6.1±0.2

GLUCOSE: Fermentable carbohydrates which provides carbon and energy

PEPTONE: This provides minerals, vitamins, nitrogen and amino acid which is essential for growth.

CHLORAMPHENICOL: An antibiotic which aids in isolating pathogenic fungi from heavily contaminated materials, as it inhibit most contaminating bacteria.

CHROMOGENIC MIX: It allows the identification and differentiation of all the species by producing easy to read result in one plates.since they present different colored colonies

BACTERIOLOGICAL AGAR: Solidifying agents

NOTE: The medium should not over heat and autoclave. The medium should free flowing homogeneous and light beige in color. If there is any physical change in medium discard and freshly prepared.

PROCEDURE

- Suspend 4.5g of medium, with distilled water and mix well.
- Dissolve the medium by heating with frequently agitation.
- Boil for one minute (Do not over heat and autoclave).
- Dispense the medium into sterile petridishes and allow solidifying.
- The colour of the medium should be clear amber slightly opalescent.
- After solidication of medium, streak the sample on the surface of agar.
- Incubate at 37°c for 24 to 48 hours.

INTERPRETATION

On Candida chromogenic agar plate the species of *Candida albicans* is differentiated by the colour of colonies.

- *Candida albicans* - green colour colonies
- *Candida trophicalis* - blue colour colonies
- *Candida krusie* - purple colour colonies

RESULT

The green colour colonies appeared on the surface of agar indicates the presence of Candida.

DISCUSSION

Candida chromogenic agar is differential and selective media for *Candida* species. The chromogenic substrate present in the media helps to differentiate the *Candida* species. The colonies with green pigmentation indicate the presence of *candida albicans.*

EXPERIMENT NO: 3

ANTIBIOTIC SENSITIVITY TEST DISC PREPARATION

AIM:

To prepare the antibiotic impregnated filter paper disc

PRINCIPLE

The antimicrobial disc to be included in the sensitivity test will depends on the pathogen and the specimen. The antimicrobial disc holding volume obtained by the blotting method. Many antimicrobial discs are commercially available with the desired volume and the concentration required according to the need.

MATERIALS REQUIRED:

- Filter paper
- Punching machine
- Micro pipette
- Test antibiotic

PROCEDURE:

- Punch out disc of 5.6mm in diameter from a sheet of filter paper or blotting paper.
- Place the disc in a pertridish and allow a distance of 2mm between each disc and sterilized in a hot air oven at 160°c for one hour.
- After allowing the disc to cool pipette out 10 or 20 µL of sterile antibiotic solutions and coat on each disc.
- The discs are dried in an incubator at 35-37 °c for 1hour.
- Stored the disc in labeled air light container in the refrigerator.

EXPERIMENT NO: 4a

ANTIBIOTIC SENSITIVITY TEST - KIRBY BAUER METHOD

AIM:

To become acquainted with the Kirby bauer technique for the evaluation of antimicrobial activity of chemotherapeutic agents.

PRINCIPLE:

Available chemotherapeutic agents vary in the scope of antimicrobial activity. Some limited spectrum of activity being effective agent only one group of the micro organisms. Others exhibits a broad spectrum of activity against a wide range of many pathogens but it is some time necessary to use several agents to determine the drug of choice.

A standardised filter paper disc agar diffusion procedure, known as kirby bauer method is frequently used to determine the drug susceptibility of micro organisms. Filter paper disc of uniform size were impregnated and then placed on the surface of agar plate that have been uniformly inoculated with actively growing log phase culture of the test organisms (lawn culture).

The confluent growth on the plate except at the zone of clearance around the antibiotic disc which inhibits the growth of the organism indicates the sustainability of the organism. The medium of choice is Muller Hinton agar with a pH of 7.2-7.4 which is poured into the plate to a uniform depth of 5mm and 25-20mm left for solidification.

MATERIALS REQUIRED:

- Culture: 0.85% saline suspension of *E. coli* and *S.aureus*
- Media:muller Hinton agar
- **Antibiotic disc:**

a) Penicillin G : 10 µg
b) Streptomycin : 10 µg
c) Tetracycline : 30 µg
d) Gentamycin : 10 µg

- **Others**: forceps, sterile cotton swab, glass wares, marking pencil and Zone measuring scale

PROCEDURE:

- Muller Hinton agar plate were prepared and sterilized.
- Then the agar surface was inoculated with the test organisms by lawn culture (pipette out 0.1 µL of culture and spread using sterile swab).
- Using forceps (sterile) antibiotic disc were placed on the surface of the agar plates with required distance and the disc were gently pressed on the agar surface.
- All the culture plate were inverted and incubated after 24 hours at 37°c.
- A zone of clearance was observed and the diameter of the zone of clearance was measured in millimetre.

RESULT:

There was growth up to the disc on the Muller Hinton Agar plate while some of had a definite zone of inhibition around the disc. Based upon the diameter of zone sensitive, resistance and intermediate of the antibiotic were recorded and tabulated.

DISCUSSION:

The agar plates that had growth rate up to the disc shows resistance of the organism while those with a definite zone of inhibition around the disc shows sensitive susceptibility of the organism to a drug is determined by the size of zone. Which itself is depends on the visible such as,

- The antibiotic and rate of diffusion of the antibiotic into the medium and its interaction with test organisms.
- Concentration of organism in the culture.
- The growth rate of organism in the culture.
- The moisture rate and incubation conditions organism.

ANTIBIOTIC SUSCEPTIBILITY TEST

ANTIBIOTIC DISC	ZONE OF INHIBITION IN MILLIMETER	SUSCEPTABILITY
Streptomycin		
Kanamycin		
Bacitracin		
Penicillin		
Erythromycin		

EXPERIMENT NO: 4b

ANTIBIOTIC SENSITIVITY TEST - STOKES METHOD

AIM:

To become acquainted with the stokes disc diffusion techniques for the evaluation of antimicrobial activity of chemotherapeutic agent.

PRINCIPLE:

In the stokes disc diffusion techniques both the test control organisms are incubated on the same plates using an inoculums which give semi confluent growth neither two heavy two light.

This means that the activity of each disc is controlled. The inhibition zone of test organisms can be compared directly with that of the control. The stokes method can be used depending on the growth of the organisms.

MATERIALS REQUIRED:

Culture: Saline suspension of *E. coli, Staphylococcus aureus*

Media: Muller Hinton Agar.

Antibiotic disc: Bactericin and Gentamycin

Equipment: Forceps, sterile cotton swab, glassware's, marking pencil.

PROCEDURE:

- Muller Hinton agar plates were prepared.
- The agar surface was inoculated with the organism in blank pattern and lawn cultured.
- *E. coli* is inoculated in the middle of the plate, *Staphylococcus aureus* is inoculated at the top and bottom. Allow the plates to dry for few minutes.
- Using a sterile forceps the antibiotics are kept in such a way that is equally touches both the organisms.
- The plates were incubated at 37°c for 24hours.
- The zone of incubation was observed, their radius is measured and compared.

RESULT:

On compared the zone of radius of the test the control organisms the result were tabulated.

ANTIBIOTIC SUSCEPTIBILITY TEST

ANTIBIOTIC DISC	*E.coli*		*S.aureus*	
	ZONE OF INHIBITION	SUSCEPTABILITY	ZONE OF INHIBITION	SUSCEPTABILITY
Streptomycin				
Kanamycin				
Bacitracin				
Penicillin				
Erythromycin				

EXPERIMENT NO: 5a

MIC DETERMINATION BY BROTH DILUTION TECHNIQUE

AIM:

To determination the smallest amount of antibiotic required to inhibit the growth of the organism by *in vitro* methods. This amount is referred as minimal inhibitory concentration (MIC).

PRINCIPLE:

The effect of chemotherapeutic agent varies with target species, some idea of effectiveness of chemotherapeutic agent against a pathogen can be obtained from minimal inhibitory concentration. MIC is the lower concentration drug that prevents pathogens growth. A pathogen should have MIC value low enough to be destroyed by the drugs. A pathogen with to high MIC value is resistant to the concentration.

MATERIALS REQUIRED:

Nutrient broth, inoculation loop, Streptomycin, pipette, spirit lamp, Test tube, log phase of *Staphylococcus* sps. (Any bacteria)

PROCEDURE:

- Antibiotic to be tested were prepared at various concentration.
- 5ml of nutrient broth was taken and 2 mg of Streptomycin was added in first tube.
- Second tube contains 5ml of nutrient broth and 1mg of Streptomycin.
- Third tube contains 5ml of nutrient broth and 0.5mg of Streptomycin.
- Each tube with One drop (0.1) of staphylococcus culture was incubated at 37°c for 24 hours.
- Turbidity was Measured of optical density (OD) a tube was prepared between the antibiotic and turbidity.

RESULT:

The Antibiotic concentration which shows _____ inhibition (MIC) for the given organism is _____**µg/5ml** it is tabulated

Organism	**Streptomycin**	
	MIC (µg/5ml)	**SUSCEPTABILITY**
S.aureus		

EXPERIMENT NO: 5b

MIC DETERMINATION BY FILTER PAPER DISC METHOD

AIM

To determine susceptibility of microorganism to antibiotic by filter paper disc method

PRINCIPLE

Filter paper disc plate method is highly standardized techniques for testing the drug sensitivity of microorganism. One can calculte the size of the zone of inhibition with MIC of the drug for the test organism. It is possible to determine whether the micro organism is resistant or susceptible to the antimicrobial agent

MATERIAL REQUIRED:

- Sterilized whatsmann no 1 filter paper disc
- Dimethyl sulphoxide (DMSO)
- Antibiotic
- Muller Hinton Agar
- 24 hrs culture of *E. coli* (any bacteria)
- Forceps
- Micropipette

PROCEDURE:

- 10mg of test antibiotic compound was weighted dissolve in 10ml of DMSO in the first tube
- 5mg of test antibiotic compound was taken dissolve in 10ml of DMSO in second tube
- 2.5mg of test antibiotic compound was taken dissolve in 10ml of DMSO in third tube
- Sterile Muller hinton agar plates were prepared.
- The Antibiotic prepared at above concentration was coated by adding to sterile filter paper disc of holding capacity 10ml using Micropipette.
- Filter paper disc with DMSO was used as control
- 0.1mg of *E. coli* culture was inoculated and own culture was performed
- The forceps was flamed and disc the various concentrations was placed
- Incubated 37°c for 24hrs in their zone of inhibition was measured

RESULT:

The Antibiotic concentration which shows _____ inhibition (MIC) for the given organism is _____**µg/10ml** it is tabulated

Organism	**Streptomycin**	
	MIC (µg/10ml)	**SUSCEPTABILITY**
E.coli		

EXPERIMENT NO: 6a

IODINE WET MOUNT PREPARATION OF PARASITES

INTRODUCTION

Stool is the most common specimen submitted to the diagnostic laboratory; the most commonly performed procedure in parasitology is the ova and parasite (O &P) examination.

Gastro-intestinal infestations (infections) by parasites (Protozoan/Helminthes) are primarily diagnosed by detecting live motile trophozoites (for protozoans); cyst (inactive dormant stage of Protozoa) or eggs (in case of Helminthes) in stool. Microscope slides made from faecal specimen of the patients can be examined under low and high power. These etiological agents are identified based on their morphological/staining (bile stained or not bile stained; in case of eggs of helminthes) characteristics**.**

Iodine Solution is often used in wet mounts of concentrated fecal material. It is useful for staining glycogen and making nuclei visible in protozoan cysts. Protozoan cysts correctly stained with iodine contain yellow-gold cytoplasm, brown glycogen material, and paler refractile nuclei. Dobell and O'Connor's Iodine is similar in formula and function to D'Antoni's Iodine.

A weak iodine solution such as Dobell and O'Connor (diluted Lugol's) is recommended for use in staining protozoan cysts. This preparation is a 1:5 dilution of Lugol's Iodine.

AIM

To examine iodine wet mount preparation of parasites in stool specimen.

MATERIALS REQUIRED

- Microscope
- clean slide
- Coverslip
- Wooden applicator.
- Lugol's iodine
- Pens or markers for labelling.

PROCEDURE

- Place a drop of Lugol's iodine solution on a slide.
- Pick up a small amount of feacal material on an applicator stick. Especially from the bloody and or mucoid areas for selection of the proper areas.

- Emulsify in the iodine solution and cover with a cover slip.
- Examine on low and high power as describe in the previous procedure.

NOTE: The film may be made directly with iodine or the iodine may be added to a saline mount by adding a drop to the edge of the cover slip so that it gradually diffuses into the saline mount.

RESULT AND DISCUSSION

Cyst, trophozoites of *Entamoeba histolytica* was observed and identified from the given sample.

EXPERIMENT NO: 6b

SALINE WET MOUNT PREPARATION OF PARASITES

INTRODUCTION:-

Faeces are the most frequent specimens collected and examined for demonstration of parasites of the gastrointestinal tract. In a parasitology laboratory, routinely, two preparations of each specimen are usually made on each slide: one unstained preparation and another temporarily stained preparation. The saline wet mount is an unstained preparation made by using physiological saline. The advantage of saline preparation is that it helps to demonstrate the motility of trophozoites. However, it does not contribute much to the definitive diagnosis of cysts or trophozoites, because internal structures are often poorly visible. To overcome this disadvantage, several stain solutions have been used for preparation of temporarily stained wet mounts of faecal specimens.

The advantages of direct wet mount include the following:

(i) It is a fast, simple procedure and provides a quick answer when positive

(ii) It provides an approximation of the parasitic burden

(iii)It can be used with unpreserved specimens to detect the characteristic motility of trophozoites

(iv)It can be used as a safeguard, as some protozoa may at times not concentrate properly because of unknown factors

(v) It can detect the motile trophozoites stage of the protozoan species

Saline wet mount is made by mixing a small quantity (about 2 mg) of faeces in a drop of saline placed on a clean glass slide. The smear is then examined under microscope. Saline wet mount is used for the detection of trophozoites and cysts of protozoa, and eggs and larvae of helminthes. It is particularly useful for detection of live motile trophozoites of *E. histolytica*, *Giardia lamblia* and *Balantidium coli.*

AIM:-

To examine saline wet mount preparation of parasite in stool sample.

MATERIALS REQUIRED:-

- Microscope
- Clean slide

- Wooden applicators.
- Normal saline solution (0.85% Sodium chloride in Distilled water)
- Pens or marker's for labelling.

PROCEDURE :-

- Place a drop of saline on the slide.
- Pick up a small amount of faecal material from several different areas. Especially from the bloody and or mucoid areas.
- Emulsify in the saline and cover with a cover slip and examine on low and high power.

RESULT AND DISCUSSION:-

From the given sample trophozoite of *Entamoeba histolytica* was observed and identified.

EXPERIMENT NO: 7a

IDENTIFICATION OF PARASITES - FORMAL ETHER CONCENTRATION

AIM

To identify the cyst, trophozoites, eggs, larvae of intestinal on the basics of morphology and internal structure.

PRINCIPLE:

The number of parasitic forms of both protozoan and/or helminthic parasites) in fecal specimens is often too low to be observed microscopically in direct wet mounts or in stained smear preparation.In such cases, the use of concentration techniques increases the chances of detecting parasitic organisms, thus increasing the sensitivity of copromicroscopic techniques. The two most commonly used stool concentration techniques are sedimentation and flotation.

Sedimentation techniques are preformed commonly in general diagnostic laboratories because they are easier to perform and less prone to technical errors**.**

Sedimentation techniques use solutions of lower specific gravity than the parasitic organisms, thus concentrating the latter in the sediment.

It takes advantage of the high specific gravity of protozoan cysts and helminth eggs compared to water. Their natural tendency to settle out in aqueous solutions can be accelerated by light centrifugation.

Formalin fixes the eggs, larvae, oocysts, and spores, so that they are no longer infectious, as well as preserves their morphology. Fecal debris is extracted into the ethyl acetate phase of the solution. Parasitic elements are sedimented at the bottom.

MATERIAL REQUIRED

A. **Reagent**

1. Ethyl acetate
2. Formalin (5 or 10 % buffered or non buffered or SAF)
3. 0.85% NaCl
4. Lugal's iodine

B. **Apparatus**

- Funnel

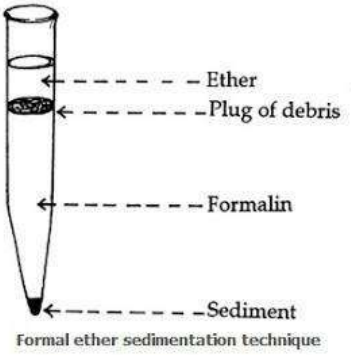


Fig-1: Formal Ether Sedimentation Technique

Formal ether sedimentation technique

- Guaze
- Centrifuge tube (15ml)
- Applicator stick
- Glass slide (1by 3 in or large)
- Cover slip (22 by 22mm ; no.1 or larger)
- Disposable glove and plastic pipette

PROCEDURE:

- Transfer half teaspoonful of feaces in 10 ml of water in a glass container and mix thoroughly.
- Place 2 layer of guaze in a funnel and strain the contents into a 15 ml centrifuge tube.
- Centrifuge for 2 minute at about 500 rpm.
- Discard the supernatant and resuspend the sediment in 10 ml of physiological saline centrifuge at 500 rpm and discard the supernatant.
- Resuspend the sediment in 7 ml of 10 % formaldehyde (1 part of 40% formalin in parts of saline)
- Add 3ml of ether (or Ethyl acetate)
- Close the tube with a stopper and shake vigorously to mix. Remove the stopper and centrifuge at 500 rpm for 2 minute
- Reset the tube in a stand four layer now become visible the top layer consist of ether second is a plug of debris and 3rd is a clear layer of formation and the sediment.
- Detach the plug or debris from the side of the tube with the aid of a glass rod and pour off the liquid leaving a small amount of formation for suspension of the sediment.
- With a pipette, remove the sediment and mix it with a drop of iodine. Examine under microscope.

RESULT AND DISCUSSION

The cyst of *Trichuris trichura* was observed and identified from the given sample.

EXPERIMENT NO: 7b

IDENTIFICATION OF PARASITES - FLOTATION METHOD

AIM:

To ensure detection of all possible organisms examine both the surface film and the sediment.

PRINCIPLE:

The flotation procedure permits the separation of protozoan cyst and certain helminth egg from excess debris through the use of a liquid with a high specific gravity. The parasite elements are recovered in the surface film and the debris remains in the bottom of the tube. The specific gravity of zinc sulfate maybe increased although this is usually causes more distortion in the organism present and it is not recommended for routine clinical use.

MATERIAL REQUIRED:

A. **Reagents**: Formalin (5 Or 10% bufferd or non buffered or SAF) 0.85%Nacl, Zinc sulfate, 33% aqueous solution.

B. **Supplies**: Finely guaze, centrifugation tube (15ml) application stick, glass slides (1 by 3m or layer) coverslips (22 by 22mm no:1 or larger) disposable glass or plastic, pipettes wire loop(bacteriology graduated cylinder).

PROCEDURE:

- Plate about one ml of faeces in a container which is feat blotted and has a diameter of less than 1 1\2 inches and capacity about 15 -20 ml.
- Add few drop of saturated salt solution (specific gravity 1,200) and stir it to make a fine emulsion.
- Add more salt solution so that the container in nearly full stirring the solution throughout.
- Add remove any coarse matter which floats op polar the container on a level surface do the final filling by a dropper until a conversation meniscus is formed.
- A glass slide 3x2 is carefully laid on the top the container so that the centre is in contact with the fluid.
- Preparation is allowed to strand for 20 minutes after which the glass slide is quickly lifted turned over smoothly as to avoid spoiling of fluid and examined under microscope after putting a coverslip.

RESULT:

Trophozoites, cyst and helminthic of egg of *Ascaris lumbricoides* were observed and identified from the given sample.

EXPERIMENT NO: 8

MORPHOLOGICAL EXAMINATION OF FUNGI IN TISSUE

INTRODUCTION

Teases mounts are used primarily for fungi wet mount are appropriate for yeast like organism. The primary purpose of preparing a tease mount is to be demonstrating conidia or other reproductive structure or morphological forms which might give information towards the identification of organism. The definite identification is based on the characteristic shape, arrangement of conidia. However the shape of hyphae also provides helpful information. The large ribbons like hyphae of zygomycetes are easily recognised while small hyphae 1 to 2 µm in size may suggest the presence of one of the dimorphic fungi.

The fungi may be prepared for microscopic observation using several techniques. The procedure traditionally used by most laboratories is the tease mount preparation. This can be done easily and quickly and is often significant to identify many of the fungi commonly encounter in the clinical laboratory. A major disadvantage of the tease preparation is that the characteristic arrangements of conidia may be disrupted when pressure is applied to the coverslip.

The easiest, most economical and suitable method for preparation are scotch tape method. The transparent tape preparation allows the observation of organism microscopically as it has grown in culture. The conidia are usually intact and the microscopically identification of an organism can be made with tease. However if the tape is not, pressed firmly enough to the colonies surface, the sample may, not be adequate for an identification.

AIM

To observe the microscopic morphology of the fungal tissue.

MATERIALS REQUIRED

- Wire bent at a 90°angle
- Bacteriologic loop holder
- Bunsen burner
- Slide and coverslip
- Lactophenol cotton blue
- Clear cellophane tape
- Teasing needle

- Tissue specimen

PROCEDURE

TEASE PREPARATION

- Place one drop of LPCB stain on a clean glass slide with a flamed and cooled bent wire pick up a small portion of fungal colony, Avoid taking the centre or outer edge is so found that reproductive structure may not yet have formed.
- Place the fungal portion in LPCB and with a second teasing needle; tease apart the hyphae so that they form a thin layer.
- Apply the coverslip and carefully press down laid to spread out the fungus, examine under microscope for reproductive structure.
- A permanent preparation may be made by trimming the edges of coverslip with clear nail polish or per mount.
- Label the slide and after examine and draw the microscopic morphology

SCOTCH OR CELLOPHANETAPE PREPARATION

- Place one drop of LPCB on an properly labelled glass slide
- Touch the adhesive slide of a small length of transparent step of the surface of the colony
- Adhere the length of the tape to the surface of the microscopic slide.
- Observe microscopically for the characteristics shape and arrangement of Conidia.

RESULT AND DISCUSSION

TEASE PREPARATION

From the morphology of the given fungus it is identified as *Fusarium sps.*

CELLOPHANE TAPE PREPARATION

From the morphology of the given fungus it is identified as *Aspergillus sps*

EXPERIMENT NO: 9

CULTIVATION OF VIRUSES-EGG INOCULATION

Introduction:

Like animals, embryonated chicken egg also posses highly specialised tissues and organs are frequently utilised to grow various viruses particularly those infecting chickens other birds. The usual routes of inoculation in chicken embryos are yolk sac method. Chorioallantoic membrane method, allantoic cavity routes, routes of inoculation in chicken embryo depends upon the various to be cultivated. Cultivation of virus embryonated egg is cultivated flock may carry anti bodies in yolk which maybe interfere in the growth of specific viruses therefore should be used.

Some factor which affect the multiplication of viruses in chicken egg are age of embryo, route of inoculation, concentration and volume of inoculums, temperature of inoculation and time of incubation. After inoculation primarily incubation temperature may be 38°C and 37°C after incubation.

Egg should be incubated by several routes section of the routes depends upon the virus and it's affinity to grow in certain tissues the routes of inoculation includes

1. Yolk sac incubation 5 to 7days - blue tongue rabies.
2. Chorioallantoic membrane 10 to 13 days -fowl pox, herpes virus.
3. Allantoic cavity route 9-11 days - new castle disease
4. Amniotic cavity route 10-12 days influenza virus

   After virus cultivation, the embryonated egg of incubated and examined daily by candling method. It embryos die within 24hrs the death is considered on specified such eggs remove from the incubator and this labelled. Some viruses like new castle disease virus kill the embryos within 2-3days.In other cases the egg are allowed to incubate up to 5-6 days. The eggs are turned upside down on primary isolation. Some viruses may not kill the embryos are produce various pathological changes the first one or two incubation and confirm whether some viruses are responsible for the incubation repeated serial bind passage are given in the egg before discarding them as negative after few passages the viruses may willing the embryos are produce other changes.

   The pathological changes are embryonated eggs are:

- Death of embryos
- Outing and developing embryos
- Haemorrhage of subcutaneous tissues
- Pick lesions on chorioallantoic membrane and thinking of chorioallantoic membrane
- Development of inclusion bodies in cytoplasm or nucleolus of infected cell.

All eggs should remain in vertical (blunt end up) those prepared for CAM. Immediately after the death of embryos or after fermentation of inoculation period the eggs should be removed from the incubator & chilled several hours before collection of embryos or other materials.

INOCULATION OF EMBRYONATED CHICKEN EGGS WITH NORMAL SALINE

1. YOLK SAC METHOD

MATERIALS REQUIRED

Seven days incubated chicken egg, normal saline, egg drilling machine, egg candles, syringes, forceps, scissors, petridish, tincture of iodine and melted paraffin.

PROCEDURE:

- Candle the egg and the yolk sac with pencil make a mark on the shell at about middle of the yolk sac.
- Drill a small hole through the egg shell at the mark without picking a shell membrane.
- Apply tincture of iodine to the hole and allow it to dry. Inoculate 0.5 ml NSS with syringes, the needle should be inserted full length through the hole before depositing the inoculum withdrawing.
- Seal the hole with sterile method paraffin and reincarnated the egg in egg incubator and examine daily by candling for 3 to 4 days.
- The yolk is harvested with the help of 5 to 10 ml syringes after apply disinfecting to shell over air sac remove shell to a distance of about 8 to 10 mm from the top of the air sac remove shell membrane and CAM from base of air sac of eggs. The allantoic fluid is collected then the embryo is placed by using forceps suspended with yolk sac, the yolk sac is opened by using scissors is sterilized petridish, the method is done in aseptic condition.
- Record observation regarding the death of embryos and after pathological lesions if any.

2. ALLANTOIC ROUTE:

MATERIALS REQUIRED:

- Embryonated chicken egg incubated for 10 days, egg incubator, drill machine, egg candle, NSS, syringe, needle, forceps, scissors, petridishes, tincture of iodine and melted paraffin.

PROCEDURE:

- While candling the egg mark on area of sac and make another mark on upper end of air sac of the egg.
- Drill a hole at the mark on the upper end of the air sac through the shell disinfects the shell on the drilled hole with sterile precautions.
- Inoculate 0.2µL NSS through the hole using 1ml syringe seal the hole with melted paraffin and incubated the egg for 4-5 days.
- For collection of allantoic fluid apply disinfection to shell over air sac break the shell to a distance of about 8-10mm from top of air sac remove the shell membrane and CAM from the Base of air sac of egg.
- White the help of 10ml syringe collects about 5 ml allantoic fluids from the cavity through air sac opening and expel the fluid in a container record observation regarding the death of embryos other pathological lesion if any.

3. AMNIOTIC ROUTE:

MATERIALS REQUIRED:

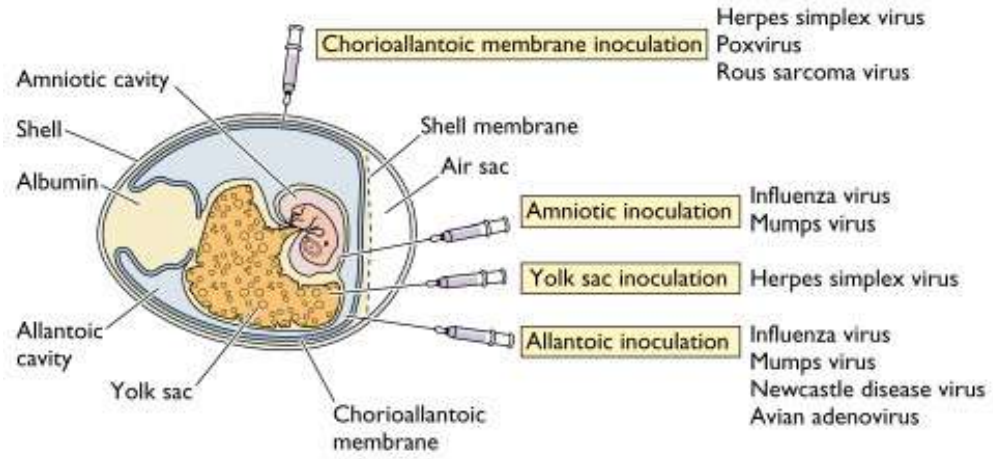
Embryonated chicken egg incubated for 10-12 days egg incubator drill machine, egg candle, nss, strings needles, forceps, scissors, petridish, iodine melted paraffin.

PROCEDURE:

- While candling the egg mark on area of air sac make another mark on the upper end of the air sac of eggs.
- Drill a hole at the mark on the upper end of the air sac through the shell in the side of egg which contain embryo disinfected the shell on the drilling hole with sterile precaution.
- Seal the hole with method paraffin incubator the egg for harvesting amniotic fluid, apply disinfection to the shell over air sac break the shell to a distance of about 8-10mm

from the top of the air sac remove Shell membrane CAM from the base of air sac of egg.

- With the help of 10ml syringe, collect about 5 ml allantoic fluids from the cavity through air sac opening and expel the fluid in a container. The embryo is placed the amniotic fluid pressure s collected from the delicate membrane surrounding the embryo using strings expel in container.
- Record observation regarding the death of the embryo other pathological lesions if any.



4. CHORIOALLANTOIC MEMBRANE ROUTE (CAM)

MATERIALS REQUIRED:

10-13 days old embryonated chicken egg, egg incubator, drill machine, egg candle, NSS, syringe & needle, forceps, scissors, pertridish, tincture of iodine, paraffin

PROCEDURE:

- Candle the egg & mark the position of embryo keep the egg in horizontal position with embryo upper most mark equatorial triangle on one side or with each side about 1cm
- Cut the egg shell at the mark the triangle without piercing through the shell membrane also mark printed cut areas of the shell & allow to dry.
- Allow disinfectant on the cut through the shell over the air sac.
- Remove the shell over the triangle with the help of the needle or forceps to expose intact shell membrane.

- With a needle the shell membrane over the air sac & on the side in the triangle without piercing the chorioallantoic membrane.
- Create a straight vacuum with a small rubber bulb at the hole over the air sac by sucking the air through the bulb. Air will pass through the opening in the shell membrane on the side of the egg allowing the CAM to drop from the shell membrane under the triangle. The air sac area will occupied by embryo membrane & fluid created on the side of the egg.
- Use 1 ml of syringe & deposit 0.2ml inoculum through the shell membrane over artificial air cell on CAM.
- Close the triangular opening in the shell with a suitable sized adhesive shell with a suitable sized adhesive tape also seal the hole over air sac space with melted paraffin or adhesive tape.
- Incubate the egg in egg incubator and examine and turn them daily for 3-4 days.
- To collect the CAM apply disinfectant on the shell over artificial air cell. Remove the shell membrane over artificial air cell with forceps to expose CAM. Cut artificial air cell portion of CAM using scissors clean the membrane with NSS in a pertridish and place the membrane in a container.
- Record observation regarding the death of the embryos and other pathological lesion if any

EXPERIMENT NO: 10

ISOLATION OF COLI-PHAGE FROM SEWAGE USING MEMBRANE FILTER TECHNIQUE

INTRODUCTION:

Coliform bacteria are relatively heamless microorganism that lives large number in the intestine of mammals, where they aid in the digestion of food. *Escherichia coli* is a common fecal coliform bacteriaum. The presence of fecal coliform bacteria in water indicates that it has been contaminated with human or other animal feces and that the potential risk exist for those who used the water raw, untreated sewage contain large number of *E.coli*. Therefore there will be raw sewage as a source of bacteriophage that infects *E coli*.

AIM:

To isolate coliphages from the given sewage sample.

PRINCIPLE:

This method include in

1. Amplify (increase number of) phage in the sewage sample by allowing them to infect and reproduce within fresh *E.coli*.
2. Collect the phages from the culture by centrifugation and filtration.
3. Detect the titer the amplified isolated phage using a plaque assay is based on the fact that each plaque on a lawn of bacteria, although it contain 106 to 107 visions along with bacterial debris represent a single infecting phase that entered me cell at the start of culture.

The infection then spread as the viruses reproduced and cell lysed eventually forming a visible plaque. The titre of a phage suspension therefore is determined by counting the number of plaque that forms a given volume of suspension. Phage titre is expressed as plaque forming units (PFU) per milliliter (1ml).

MATERIALS REQUIRED:

- Overnight culture of *E.coli*
- Nutrient Agar plate
- Sewage sample
- Test tube
- Pipettes

- Membrane filter
- Filtrate apparatus.

PROCEDURE:

ENRICHMENT TECHIQUE:

- About 45 ml of Sewage was taken in a sterile flask and to this 5 ml of 10X nutrient broth was added.
- Aseptically 5 ml of *E.coli* (12- 24 hours old culture) was added to flask containing sewage.
- Flask was mixed gently and incubated for 24 hours at 37ºC.

FILTERATION:

- About 10ml of enrichment culture was taken and centrifuged and 5000rpm for 10min to remove the bacterial cell.
- The supernatant was filtered by passing into membrane (0.45um in diameter).
- Now the filtered contain only phages.

SEEDING:

- Soft nutrient Agar tubes was taken and liquefied cooled at 50 ºC.
- The tubes were kept in water bath to maintain the tube at 50 ºC to prevent solidification.
- About 4-5 drop off filtrate was added to the soft agar tubes.0.3 ml of *E.coli* culture was added to the soft agar tube and mixed by Rolling that tubes between the hands.
- The content of the soft agar tube was third poured over the Nutrient Agar plates.
- Once the soft agar is solidified and the plates were inverted and incubated for 24 hours at 37 ºC.
- The plates were observed for the plaque formation.

RESULT & DISCUSSION:

Clear lytic areas are seen on the plates *E.coli* has grown into a lawn clear lytic area indicates the presence of phages.

EXPERIMENT NO: 11

EXAMINATION OF PLANT DISEASE

POTATO WILT DISEASE

Verticillium wilt, also known as potato wilt, is a fungal disease that can be caused by either Verticillium dahliae or Verticillium alboratrum. Both of these fungi can survive in the soil, in infected plant parts and seed pieces for a long time. In fact, Verticillium dahliae has been found to remain in soil for up to seven years. Wilt can result in a reduction in tuber size and stem-end discoloration. The fungus attacks the potato plant through the roots and interferes with the transportation of water. Potato plants exhibit disease symptoms when they turn yellow prematurely. Infected tubers may show vascular discoloration in rings near the end of the stem. Wilted potato plants eventually die.

SYMPTOMS:

- In addition to the potato, the pathogen also damages plants such as chili, tomato, tobacco and egg plant, as well as several species of weeds.
- The symptoms of bacterial wilt infection can be seen on all parts of infected plants.
- Infected plant begins to wilt, starting from the tips of the leaves or where the stems branch out, and then spreading to all parts of the plant.
- Leaves become yellow at their bases, then the whole plant wilts and dies. When stems are cut a brown coloured ring will be visible.
- When a tuber is cut in half, black or brown rings will, however, be visible. If left for a while or squeezed, these rings will exude a thick white fluid.
- A further symptom is fluid coming out of tuber eyes. This can be signified by soil sticking to tuber eyes when crops are harvested. Serious infection causes tubers to rot.

CITRUS CANKER

Citrus canker is a contagious disease of citrus (and some other plant species of the Rutaceae family) caused by the bacteria *Xanthomonas citri*. Infected trees display unsightly lesions which can form on leaves, fruit and stems. Citrus canker appears as raised spongy lesion of leaves, twigs and fruit, which gradually increase in size to 5-10mm over several months. Inoculum remains in the lesions of plants from year to year (overwintering), and are the primary source of

new infections. Bacteria can also survive on straw or mulch, or in soil. Active infections typically begin in early spring. Bacteria from lesions are spread throughout the plant by rainfall. In rain storms, bacteria can be carried between trees, up to 100m. The disease can become latent in fields for long periods, and become active again in periods of high rainfall and warm weather. The disease is not transmitted by seeds.

SYMPTOMS:

Citrus canker is mostly a leaf-spotting and fruit rind blemishing disease, but when conditions are highly favorable for infection, infections cause defoliation, shoot dieback, and fruit drop. Citrus canker symptoms include brown spots on leaves, often with an oily or water-soaked appearance and blisters like formation. The spots (technically called lesions) are usually surrounded by a yellow halo, and they can be seen on both the upper and lower sides of the leaf. Similar symptoms can appear on fruit and stems.

RICE DWARF DISESASE

The Rice Dwarf Virus was the first studied plant virus. It has since been used as a model teaching tool for understanding plant virus mechanisms. RDV uses an insect vector to infect rice and wheat plants. The virus is an isohedral double shelled virus, ranging from 70-75 nm in diameter. There are three strains of the RDV: O, D84, and S. Strain S is the most virulent and causes the most severe symptoms. The RDV is isolated to China and Japan, but the detrimental effects it has on the grain industry impacts globally.

The RDV is an isohedral virus containing 32 capsomeres. The capsomeres are arranged in a conspicuous manner. The virons are isometeric and not enveloped. The virus has an angular profile. The RDV enters the host cell then begins replication and assembly. The viruses then participate in intracellular transport by taking over host mechanisms. Tubulars are created using the host inner and outer membrane and extends the structure outward to other host cells. This enables the RDV to move intracellularly into un-infected host cells and being replication.

SYMPTOMS:

Rice plants infected with RDV show pronounced stunting increased tillering and short leaves that are darker green in colour with fine chlorotic specks. Infected plants usually survive until harvest time but rarely produce panicles. Panicles which are produced are poor and bear unfilled

grains. Infected cells contain large round or oval inclusion bodies in the cytoplasm. The bodies consist of a viroplasmic matrix and numerous virus particles. Tubules enclosing virus particles and paracrystalline bodies occur in or around the bodies. The chlorotic specks in the leaf correspond to masses of cells which are filled with inclusions and virus particles. Starch accumulates in infected rice tissues.