Semester – III

17MBU314B **(3H – 1C)**

MICROBIAL DIAGNOSIS IN HEALTH CLINIC - PRACTICAL

Instruction Hours / week: L: 0 T: 0 P: 3 **Marks:** Internal: **40** External: **60** Total: **100**

End Semester Exam: 3 Hours

SCOPE

Acquire knowledge to identify the common infectious agents with the help of laboratory procedures and use antimicrobial sensitivity tests to select suitable antimicrobial agents.

OBJECTIVES

- To provide an understanding of the natural history of infectious diseases in order to deal with the etiology, laboratory diagnosis, treatment and control of infections in the community.

EXPERIMENTS

1. Collection and processing of clinical specimen – Sputum.
2. Collection and processing of clinical specimen – Urine.
3. Collection and processing of clinical specimen – Blood.
4. Collection and processing of clinical specimen – Stool.
5. Antibiotic sensitivity testing by Kirby-Bauer method
6. Determination of minimal inhibitory concentration.

SUGGESTED READINGS

1. Brooks G.F., Carroll K.C., Butel J.S., Morse S.A. and Mietzner, T.A. (2013). Jawetz, Melnick and Adelberg's Medical Microbiology. 26th edition. McGraw Hill Publication.
2. Collee JG, Fraser, AG, Marmion, BP, Simmons A (2007) Mackie and Mccartney Practical Medical Microbiology, 14th edition, Elsevier.
3. Greenwood D, Slack R, Barer M, and Irving W. (2012). Medical Microbiology, 18th Edition. Churchill Livingstone.
4. Ryan KJ and Ray CG. (2014). Sherris Medical Microbiology, 6th Edition. McGraw-Hill Professional.

COLLECTION AND PROCESSING OF CLINICAL SPECIMEN - SPUTUM

INTRODUCTION

Human tuberculosis is principally transmitted by the inhalation of bacilli in most droplets coughed out by the individuals with open pulmonary tuberculosis. The site of initial infection is usually the lung. The bacilli are engulfed by alveolar macrophage in which they replicate to form the initial lesion. Within about 10 days of infection clones of antigen specific 7% lymphocytes are produced.

AIM

To diagnose the specimen received and to isolate the organism suspected for tuberculosis.

SAMPLE

Sputum is the most usual specimen. Apart from it bronchial washings, biopsies, gastric aspirate, CSF, pleural fluids were also collected in certain cases.

Sputum is a mixture of bronchial secretion and inflammatory exudates coughed out and expedorated in some infections. During bacterial infection sputum will be purulent and contain yellow, green and opaque materials. Usually disposable wide mouthed plastic container of about 100ml capacity is used to collect specimen.

TRANSPORT OF SPECIMEN

The specimens are usually delivered to laboratory preferably within 2 hours to prevent the drying of delicate bacterial, viral and *Mycoplasmal species* in the specimen.

SPECIMEN PROCESSING

Homogenization

The specimen is usually processed in 2 ways:

1. By means of adding sterile glass beads.
2. Equal volume of 1% buffered pancreatin was added and shaken well and was placed in water bath for an hour. The suspension was shaken well every 15 minutes and this helps in the uniform distribution of the specimen.

PROCEDURE

Microscopy

Acid fast staining (or) Ziehl Nelson staining

The heat fixed smear of specimen was flooded with carbolfuchsin and heated. Then the smear was washed with water and decolorized with acid alcohol (20% H_2So_4 and then with 95% ethanol) for 2 minutes. The smear was then counterstained with Loeffelers methylene blue and then observed under oil immersion.

Mantoux test

Graded doses of tuberculin are injected intradermally on the forearm using a tuberculin syringe.

Culture method

Lowenstein Jenson's medium

The specimen was inoculated onto two bottles of LJ medium and incubated at 37°c and inspected for growth of typical colonies weekly once.

INTERPRETATION OF RESULTS

Acid fast staining

Under the oil immersion objective, acid fast bacilli can be seen as bright red rods around the blue background.

Mantoux test

On examination after 48-72 hours edemas and indurations at the site measuring atleast 6-10mm in diameter can be seen.

Cultural methods

Lowenstein Jensons medium

Buff colored colonies will be appeared with a dry bread crumb like appearance in about 2 weeks. Isolation from clinical specimens may also take upto 8 weeks in certain cases.

Based on the observation of the microscopy, cultural characteristics and immunological techniques the possible organism in the specimen may be interpreted as *Mycobacterium tuberculosis.*

COLLECTION AND PROCESSING OF CLINICAL SPECIMEN – URINE

INTRODUCTION

Urinary tract infections occur more frequently in women than men due to shortness of female urethra. *E.coli* is the principle urinary tract pathogen. Other less common cause of UTI is *Staphylococci, Enterococci, Proteus, Klebsiella and other Coliforms and Pseudomonas aeroginosa*

Most infections originate from the patients bowel flora (endogenous infection). Infections are usually caused by the entry of microorganisms from external sources. Microorganisms at the opening of the urethra, careless personal hygiene and sexual intercourse contribute to the incidence of UTI. Opportunistic organisms present in intestine can also cause the infection.

AIM

To diagnose the specimen and to isolate the organism suspected for urinary tract infections.

SPECIMEN COLLECTION

Specimen should be collected carefully without any urethral contamination then it should be sterile.

Clean catch midstream urine

The first portion of the urine which is more likely to be contaminated must be avoided as the bacteria will have multiplied to high levels after overnight incubation in the bladder.

Clean catch of midstream urine was collected in sterile wide mouth screw cap tubes.

PROCEDURE

Microscopic examination

Wet mount method

A drop of fresh uncentrifuged urine was placed on slide and covered with cover glass and examined under light microscope.

Gram staining

Gram stained smear of urine was placed on slide and covered with cover glass and examined under light microscope

Culture methods

The given urine specimen was diluted and streaked on nutrient agar plates and CLED agar plates and the plates were incubated at 37°c for 24hrs.

RESULTS

Microscopic examination

Wet mount method

Leucocytes, epithelial cells, red blood cells, casts, yeast cells and bacteria in large number are observed. Two pus cells in high power field is an indication of UTI infections.

Gram staining

Large numbers of gram negative rods were observed.

Cultural characteristics

Nutrient agar

Colonies was enumerated and morphology was studied. Enumeration was done by counting the colonies.

1000 colonies=10,000 organisms/ml of urine was significant for UTI.

Cysteine lactose electrolyte deficient agar

Translucent blue grey colonies were observed.

Based on the observation of the microscopy, cultural characters the possible organism in the specimen was identified as *Proteus sp.*

COLLECTION AND PROCESSING OF CLINICAL SPECIMEN - BLOOD

INTRODUCTION

Enteric fever is a term that is generally used for fever caused due to interaction by *Salmonella typhi* and *Salmonella paratyphi* A *and* B. Enteric fever caused by *Salmonella species* are called as typhoid and paratyphoid fever, on the basis of species of this genera that causes fever.

AIM

To diagnose the specimen and to isolate the organism suspected for enteric fever.

SPECIMEN

Blood is the usual specimen received when enteric fever is suspected. Faeces and urine can also be received for diagnosis.

PROCEDURE

The laboratory diagnosis of enteric fever includes the following

1. Microscopic examination
2. Culture methods
3. Biochemical tests
4. Antibiotic sensitivity testing

Bacteriological diagnosis of enteric fever consists of the isolation of bacilli (*Salmonella sp*) from the patient and demonstration of antibodies in the patient serum.

MICROSCOPIC EXAMINATION

Gram staining

The specimen received was made as a smear and gram staining was performed.

Cultural methods

The specimen was inoculated into the blood agar, Macconkey agar, Xylose Lysine deoxycholate medium and then incubated for 24 hours.

Blood culture

About 5-10 ml of blood specimen was inoculated into the culture bottle containing 5-100ml of 0.5% bile broth. After overnight incubation at 37°C the bile broth was subcultured on Macconkey agar plates and the non lactose fermenting colonies were taken further for biochemical tests and motility tests.

Biochemical tests

The biochemical tests for colonies obtained from the cultural medium mentioned above were performed. The results of the biochemical tests were tabulated.

Antibiotic sensitivity test

The antibiotic sensitivity test was performed by Kirby-Bauer method using Muller Hinton agar medium.

Immunological techniques

WIDAL test

In a clean glass slide one drop of undiluted test serum was added in first 4 circles (1-4) and one drop of antigen O, H, AH, BH was added respectively. The content of each circle was mixed with separate applicator sticks and slide rocked for a minute.

RESULTS

Microscopic examination

Gram staining

Gram negative rods were observed.

Culture methods

Blood agar

Large beta hemolytic colonies were observed on the surface of blood agar plates.

Macconkey agar

Pale non lactose fermenting colonies

Xylose Lysine Deoxycholate Medium

Small red color colonies were observed.

Immunological techniques

WIDAL test

Agglutination was observed in the serum added with O and H antigens.

Based on the observation of the microscopy, cultural characters, biochemical tests and immunological techniques the possible organism in the specimen is interpreted as *Salmonella typhi*.

TABLE SHOWING LABORATORY DIAGNOSIS OF

ENTERIC FEVER

SALMONELLA TYPHI	RESULTS
Motility	Positive
Indole test	Negative
Methyl red test	Positive
Voges proskauer test	Negative
Citrate test	Negative
Urease test	Negative
TSI agar test	Alkaline/Acid
CARBOHYDRATE FERMENTATION	
Glucose	Acid
Lactose	Non fermenting
Sucrose	Non fermenting
Mannitol	Non fermenting
H_2S production test	Positive

COLLECTION AND PROCESSING OF CLINICAL SPECIMEN - STOOL

INTRODUCTION

Diarrhoea is a condition where intestinal discharge is expelled in a liquid state which is known as the stool. Diarrhea is caused by variety of microorganisms ranging from bacteria to viruses. It is accompanied by abdominal pain and discomfort, loss of water and electrolytes which are the result of diarrhoea and it leads to severe dehydration. Toxigenic organisms such as *Vibrio cholera* causes to severe dehydration *E.coli* causes travelers diarrhoea some intestinal viruses and Rota viruses can also cause diarrhea.

AIM

To diagnose the specimen and to isolate the organism suspected for diarrhoea.

SPECIMEN

Stool sample

COLLECTION AND TRANSPORT

The specimen was collected in a clean, dry, disinfectant free wide mouthed container without urine contamination. A spoonful of specimen containing mucus, blood is transferred to a clean leak proof container. When faecal sample is not available rectal swab is preferable. It can transport using sterile Cary Blair medium.

PROCEDURE

Microscopic examination

Gram staining

The specimen was made as a smear and gram staining was performed.

Motility test

Hanging drop technique was carried out using the specimen.

Culture methods

The stool sample was inoculated on various media like Macconkey agar, Thiosulphate citrate bile salt media, Xylose lysine deoxycholate agar, SS agar and incubated for 16-24 hours at 37° c and the results were noted.

Biochemical tests

The biochemical tests for the colonies obtained above were performed. The results of biochemical tests were tabulated.

Antibiotic sensitivity test

The antibiotic sensitivity test was performed by Kirby Bauer method using Muller Hinton Agar medium.

RESULT

Microscopic examination

Gram staining

Pink coloured gram negative rods were observed.

Hanging drop method-motility test

Motile rods were observed.

Culture methods

Pink colored lactose fermenting colonies were observed on Macconkey agar. The given specimen was processed and based on the observation of the microscopy, cultural characters, biochemical tests and antibiotic sensitivity test. The possible organism was identified as *Escherichia coli.*

TABLE SHOWING LABORATORY DIAGNOSIS OF DIARRHOEA

E.coli	**RESULTS**
Motility	Positive
Indole test	Positive
Methyl red test	Positive
Voges proskauer test	Positive
Citrate test	Negative
Urease test	Negative
Macconkey agar	Pink color colonies
CARBOHYDRATE FERMENTATION	
Glucose	Acid/Gas
Lactose	Acid/Gas
Sucrose	Non fermenting
Mannitol	Acid/Gas

Antibiotic sensitivity test - Kirby-Bauer Method

Aim

Evaluate the antimicrobial activity of selected antibiotics using the Kirby-Bauer method.

Principles

One method that is used to determine antibiotic susceptibility is the sensitivity disk method of **Kirby- Bauer** (named after W. Kirby and A. W. Bauer in 1966). In this method, antibiotics are impregnated onto paper disks and then placed on a seeded Mueller-Hinton agar plate using a mechanical dispenser or sterile forceps. The plate is then incubated for 16 to 18 hours, and the diameter of the **zone of inhibition** around the disk is measured to the nearest millimeter. The inhibition zone diameter that is produced will indicate the susceptibility or resistance of a bacterium to the antibiotic (figure 43.1). Antibiotic susceptibility patterns are called **antibiograms.** Antibiograms can be determined by comparing the zone diameter obtained with the known zone diameter size for susceptibility. For example, a zone of a certain size indicates susceptibility, zones of a smaller diameter or no zone at all show that the bacterium is resistant to the antibiotic. Frequently one will see colonies within the zone of inhibition when the strain is antibiotic resistant. Many factors are involved in sensitivity disk testing and must be carefully controlled. These include size of the inoculum, distribution of the inoculum, incubation period, depth of the agar, diffusion rate of the antibiotic, concentration of antibiotic in the disk, and growth rate of the bacterium. If all of these factors are carefully controlled, this type of testing is highly satisfactory for determining the degree of susceptibility of a bacterium to a certain antibiotic. The Kirby-Bauer method is not restricted to antibiotics. It may also be used to measure the sensitivity of any microorganism to a variety of antimicrobial agents such as sulfonamides and synthetic chemotherapeutics.

Materials required

1. 4 150 × 15 mm Mueller-Hinton agar plates antibiotic disk dispensers (BBL or Difco) or assorted individual vials containing antibiotic disks
2. 4 sterile swabs

3. 4- to 6-hour tryptic soy broth cultures of *Staphylococcus aureus* (ATCC 25903), *Escherichia coli* (ATCC 11229), *Pseudomonas aeruginosa* (ATCC 10145), and *Klebsiella pneumoniae* (ATCC e13883)
4. 35°C incubator
5. forceps
6. metric rulers
7. wax pencil
8. 70% ethyl alcohol and beakers
9. Bunsen burner

Procedure

1. With a wax pencil, mark the lid of each Mueller- Hinton agar plate with your name, date, and the name of the bacterium to be inoculated. Each group of students will inoculate the surface of four Mueller-Hinton plates with *S. aureus, E. coli, P. aeruginosa,* and *K. pneumoniae,* respectively. Use a separate, sterile cotton swab for each bacterium. The swab is immersed in the culture tube, and the excess culture is squeezed on the inner side of the test tube. If there are sufficient supplies, you may wish to analyze the antimicrobial sensitivity of microorganisms from your throat.
2. The swab is then taken and streaked on the surface of the Mueller-Hinton plate three times, rotating the plate 60° after each streaking. Finally, run the swab around the edge of the agar. This procedure ensures that the whole surface has been seeded. Allow the culture to dry on the plate for 5 to 10 minutes at room temperature with the top in place.
3. Dispense the antibiotics onto the plate either with the multiple dispenser or individually ith the single unit dispenser. Make sure that contact is made between the antibiotic disk and the culture by gently pressing the disk with alcohol-flamed forceps. DO NOT PRESS THE DISK INTO THE AGAR, AND DO NOT MOVE THE DISK ONCE IT IS PLACED ON THE AGAR.
4. Incubate the plates for 16 to 18 hours at 35°C. DO NOT INVERT THE PLATES.

   Measure the zones of inhibition to the nearest mm for each of the antibiotics tested. Record the results in the report for exercise 43. Use interpretation table as an aid. For each antibiotic, determine whether the bacteria are resistant or susceptible.

RESULT

On comparing zone of radius of the test and control organism, the results were tabulated.

TABLE SHOWING THE RESULTS OF ANTIMICROBIAL SENSITIVITY TEST

S. No.	Chemotherapeutic agent	*Escherichia coli*		*Staphylococcus aureus*	
		Zone	**Susceptiblity**	**Zone**	**Susceptiblity**
1.	BACITRACIN	10 mm	Intermediate	13 mm	Intermediate
2.	GENTAMYCIN	20 mm	Sensitive	19 mm	Sensitive

MIC DETERMINATIN BY BROTH DILUTION TECHNIQUE

AIM

To determine the smallest amount of antibiotics required to inhibit the growth of the organism by *invitro* method. 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 minimum inhibitory concentration. MIC is the lower concentration drug that prevents a pathogen growth; A pathogen should have MIC value low enough to be destroyed by the drug. A pathogen with to high MIC value is resistant to the agent that normal body concentration.

Materials Required

- Nutrient broth
- Inoculation loop
- Streptomycin
- Pipette
- Sprit lamp
- Test tube
- Young culture of *Staphylococcus* sps

PROCEDURE

1. Antibiotic to be tested were prepared at various concentration.
2. 5ml of nutrient broth was taken and mg of streptomycin was added in first tube.
3. Second tube contain 5ml of nutrient broth and 2-5mg of streptomycin.
4. Third tubes contain 5ml of nutrient broth and 1.25mg of streptomycin.
5. Each tube with 1drop of *staphylococcus* culture was incubated at 37°C for 24 hours.
6. Turbidity was measured in terms of optical density (OD) a tube was prepared between the antibiotic and turbidity.

RESULT

Low concentration of antibiotic will show maximum OD due to low inhibitory effect, when high concentration will reveal the maximum OD.

TABLE SHOWING OD VALUE AT 610nm

S. No.	CONCENTRATION	OD VALUE AT 610nm
1.	5mg/ml	0.26
2.	2.5mg/ml	0.51
3.	1.25/ml	0.92

Experiment No:

MIC DETERMINATION BY FILTER PAPER DISC METHOD

AIM

To determine the susceptibility of microorganism to antibiotics by filter paper disc method.

PRINCIPLE

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

MATERIALS REQUIRED

- Sterilized Whatmann No 1 filter paper disc
- Dimethyl sulphoxide
- Antibiotics(tetracycline)
- Muller Hinton agar
- 24 hours culture of *E.coli*
- Forceps
- Micropipette

PROCEDURE

1. 10mg of test antibiotic compound was weighed and dissolved in 10ml of DMSO in the first tube.
2. 5mg of test antibiotic compound was taken and dissolved it in 10ml of DMSO in second tube.
3. 2.5mg of test antibiotic compound was taken and dissolved it in 10ml of DMSO in third tube.
4. Sterile Muller Hinton agar plates were prepared.
5. The antibiotic prepared at above said concentrations were added to sterile filter paper disc of holding capacity 10ml.
6. Filter paper disc with DMSO was used as control.
7. 0.1ml of *E.coli* culture was inoculated and lawn culturing was performed.
8. The forceps was flamed and disc of various concentration were placed.
9. Incubated at 37°C for 24 hours in their zone of inhibition was measured.

OBSERVATION AND RESULT

Observe the plates after 24 hours and note the zone of inhibition around the disc which indicates the organism was inhibited by antibiotic. The zone of inhibition of *E.coli* were Measured and tabulated.

TABLE SHOWING ZONE OF INHIBITION OF *E. coli* BY TETRACYCLINE

S. No.	Chemotherapeutic agent	10mg/ml	5mg/ml	2.5mg/ml
1.	Tetracycline	18	11	7

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