16MBU412 MEDICAL MICROBIOLOGY - PRACTICAL **Semester – IV (3H – 2C)**

Instruction Hours / week: L: 0 T: 0 P: 4 **Marks:** Internal: **40** External: **60** Total: **100**
End Semester Exam: 9 Hours

SCOPE

It provides the ability to characterize, isolate and identify different microbes. It includes a detailed study of characterization, etiology, pathogenicity, clinical systems, and laboratory diagnosis of disease causing Microorganisms.

OBJECTIVES

- To introduce the knowledge of the medically important microganisms, microbial morphology with the main focuses being the characterization, isolation and identification of different microorganism.

EXPERIMENTS

1. Identify bacteria (any three of *E. coli*, *Salmonella*, *Pseudomonas*, *Staphylococcus*, *Bacillus*) using laboratory strains on the basis of cultural, morphological and biochemical characteristics: IMViC, TSI, nitrate reduction, urease production and catalase tests.
2. Study of composition and use of important differential media for identification of bacteria: EMB Agar, MacConkey agar, Mannitol salt agar, Deoxycholate citrate agar, TCBS, Salmonella Shigella/BSA Agar.
3. Study of bacterial flora of skin by swab method.
4. Antibacterial sensitivity assay by Kirby-Bauer method.
5. Determination of minimal inhibitory concentration (MIC) of an antibiotic.
6. Study symptoms of the diseases with the help of photographs: Polio, anthrax, herpes, chicken pox, HPV warts, AIDS (candidiasis), dermatomycoses (ring worms).
7. Study of various stages of malarial parasite in RBCs using permanent mounts.

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. Greenwood D, Slack R, Barer M, and Irving W. (2012). Medical Microbiology, 18th Edition. Churchill Livingstone.
3. Ryan KJ and Ray CG. (2014). Sherris Medical Microbiology, 6th Edition. McGraw-Hill Professional.
4. Ananthanarayan R. and Paniker C.K.J. (2009) Textbook of Microbiology. 8th edition, University Press Publication.
5. Madigan MT, Martinko JM, Dunlap PV and Clark DP. (2014). Brock Biology of Microorganisms. 14th edition. Pearson International Edition.
6. Goering R., Dockrell H., Zuckerman M. and Wakelin D. (2007). Mims' Medical Microbiology. 4th edition. Elsevier
7. Willey JM, Sherwood LM, and Woolverton CJ. (2013). Prescott, Harley and Klein's Microbiology. 9th edition. McGraw Hill Higher Education.

2018

List of practicals – lecture plan

S. No	Experiment name	Hours	Support materials
1.	Staining	4 weeks/ 16 hours	Manual of Medical lab technology – Dr. S. Rajan
2.	Media preparation	2 weeks/ 8 hours	Manual of Medical lab technology – Dr. S. Rajan
3.	Biochemical tests	1 weeks/ 4 hours	Manual of Medical lab technology – Dr. S. Rajan
4.	Study of normal bacterial flora	1 weeks/ 4 hours	Manual of Medical lab technology – Dr. S. Rajan
5.	Antibiotic sensitivity testing by Kirby bauer method	1 week/4 hours	Lab diagnosis of infectious disease
6.	Determination of minimal inhibitory concentration	1 week/ 4 hours	Lab diagnosis of infectious disease
7.	Study about viral infections by photographs	1 week/ 4 hours	Lab diagnosis of infectious disease
8.	Study about malaria parasite	1 week/ 4 hours	Lab diagnosis of infectious disease

Prepared by: Dr. S. DInesh Kumar, Assistant Professor, Dept of Microbiology, KAHE

2018

List of practicals

1. Staining
2. Media preparation
3. Biochemical tests
4. Study of normal bacterial flora
5. Antibiotic sensitivity testing by Kirby bauer method
6. Determination of minimal inhibitory concentration
7. Study about viral infections by photographs
8. Study about malaria parasite

References:

1. Manual of Medical lab technology – Dr. S. Rajan
2. Lab diagnosis of infectious disease

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2017

Experiment no 6 – Determination of minimum inhibitory concentration

Dilution Methods

Dilution susceptibility testing methods are used to determine the minimal concentration of antimicrobial to inhibit or kill the microorganism. This can be achieved by dilution of antimicrobial in either agar or broth media. Antimicrobials are tested in $\log_2$ serial dilutions (two fold).

Minimum Inhibitory Concentration (MIC)

Diffusion tests widely used to determine the susceptibility of organisms isolated from clinical specimens have their limitations; when equivocal results are obtained or in prolonged serious infection e.g. bacterial endocarditis, the quantitation of antibiotic action vis-a-vis the pathogen needs to be more precise. Also the terms 'Susceptible' and 'Resistant' can have a realistic interpretation. Thus when in doubt, the way to a precise assessment is to determine the MIC of the antibiotic to the organisms concerned.

There are two methods of testing for MIC:

(a) Broth dilution method

(b) Agar dilution method.

Dilution methods can be carried out in 2 ways

A. Broth dilution

Broth dilution testing allows the option of providing both quantitative (MIC) and qualitative (category interpretation) results. MIC can be helpful in establishing the level of resistance of a particular bacterial strain and can substantially affect the decision to use certain antimicrobial agents.

Broth dilution can again be performed by 2 ways

1. Macro dilution: Uses broth volume of **1 ml** in standard test tubes .
2. Microdilution: Uses about **0.05 to 0.1 ml** total broth volume and can be performed in a microtiter plate or tray .

The procedure for both macro and microdilution are same except the volume of the broth.

B. Agar dilution

MIC of an antibiotic using broth dilution method is determined by using the following procedure

1. Preparation of antibiotic stock solution
2. Preparation of antibiotic dilution range
3. Preparation of agar dilution plates
4. Preparation of inoculum
5. Inoculation
6. Incubation
7. Reading and interpreting results

Preparation of antibiotic Stock solution.

Antibiotic stock solution can be prepared by commercially available antimicrobial powders (with given potency). The amount needed and the diluents in which it can be dissolved can be calculated by using either of the following formulas to determine the amount of antimicrobial powder (1) or diluent (2) needed for a standard solution:

Prepare antimicrobial agent stock solutions at concentrations of at least 1000 μg/mL (example: 1280 μg/mL) or 10 times the highest concentration to be tested, whichever is greater.

Because microbial contamination is extremely rare, solutions that have been prepared aseptically but not filter sterilized are generally acceptable. If desired, however, solutions may be sterilized by**membrane filtration**. Dispense small volumes of the sterile stock solutions into sterile glass, polypropylene, polystyrene, or polyethylene vials; carefully seal; and store (*preferably at −60 °C or below, but never at a temperature warmer than −20 °C and never in a self-defrosting freezer).* Vials may be thawed as needed and used the same day.

Preparation of antibiotic dilution range

- Use sterile 13- x 100-mm test tubes to conduct the test. If the tubes are to be saved for later use, be sure they can be frozen.
- Close the tubes with loose screw-caps, plastic or metal closure caps, or cotton plugs.
- Prepare the final two fold (or other) dilutions of antimicrobial agent volumetrically in the broth. A minimum final volume of 1 mL of each dilution is needed for the test.

Note: For, microdilution,only 0.1 ml is dispensed into each of the 96 wells of a standard tray.

Preparation of inoculum

1. Prepare the inoculum by making a direct broth suspension of isolated colonies selected from an 18- to 24-hour agar plate *(use a non-selective medium, such as **blood agar**).*
2. Adjust the suspension to achieve a turbidity equivalent to a **0.5 McFarland turbidity standard**. This results in a suspension containing approximately 1 to 2 x 10^8 colony forming units (CFU)/mL for*Escherichia coli* ATCC®a 25922.
3. Compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines.
4. Optimally within 15 minutes of preparation, dilute the adjusted inoculum suspension in broth so, after inoculation, each tube contains approximately 5 x 10^5 CFU/mL.**Note:** *This can be accomplished by diluting the 0.5 McFarland suspension 1:150, resulting in a tube containing approximately 1 x 10^6 CFU/mL. The subsequent 1:2 dilution in step 3 brings the **final inoculum to 5 x 10^5 CFU/mL.***

Minimum Bactericidal Concentrations(MBC)

The main advantage of the 'Broth dilution' method for the MIC determination lies in the fact that it can readily be converted to determine the MBC as well.

Method

Dilutions and inoculations are prepared in the same manner as described for the determination of MIC. The control tube containing no antibiotic is immediately subcultured (Before incubation) by spreading a loopful evenly over a quarter of the plate on a medium suitable for the growth of the test organism and incubated at 37^oC overnight. The tubes are also incubated overnight at 37^oC. Read the MIC of the control organism to check that the drug concentrations are correct.

Note the lowest concentration inhibiting growth of the organisms and record this as the MIC. Subculture all tubes not showing visible growth in the same manner as the control tube described above and incubate at 37^{o}C overnight. Compare the amount of growth from the control tube before incubation,which represents the original inoculum. The test must include a second set of the same dilutions inoculated with an organism of known sensitivity .These tubes are not subcultured; the purpose of the control is to confirm by its MIC that the drug level is correct, whether or not this organism is killed is immaterial.

Reading of result

These subcultures may show

- Similar number of colonies- indicating bacteriostasis only.
- A reduced number of colonies-indicating a partial or slow bactericidal activity.
- No growth- if the whole inoculum has been killed
- The highest dilution showing at least 99% inhibition is taken as MBC

Micro-broth dilution test

This test uses double-strength Müeller-Hinton broth, 4X strength antibiotic solutions prepared as serial two-fold dilutions and the test organism at a concentration of $2x10^6$/ml. In a 96 well plate, 100 µl of double-strength MHB, 50 µl each of the antibiotic dilutions and the organism suspension are mixed and incubated at 35°C for 18-24 hours. The lowest concentration showing inhibition of growth will be considered the MIC of the organism.

Reading of result

MIC is expressed as the highest dilution which inhibited growth judged by lack of turbidity in the tube. Because very faint turbidity may be given by the inoculum itself,the inoculated tube kept in the refrigerator overnight may be used as the standard for the determination of complete inhibition. Standard strain of known MIC, run with the test is used as the control to check the reagents and conditions.

Minimum inhibitory concentration (MIC) is determined when a patient does not respond to treatment thought to be adequate, relapses while being treated or when there is immunosuppression.

Medical Microbiology Spotters

1. 

Biohazard refers to any biological materials(microorganisms, plants, animals, or their byproducts) that pose a threat to the health of living organisms.

- **Human blood and blood products.** This includes items that have been contaminated with blood and other body fluids or tissues that contain visible blood.
- **Animal waste.** Animal carcasses and body parts, or any bedding material used by animals that are known to be infected with pathogenic organisms.
- **Human body fluids.** Semen, cerebrospinal fluid, pleural fluid, vaginal secretions, pericardial fluid, amniotic fluid, saliva, and peritoneal fluid.
- **Microbiological wastes.** Common in laboratory settings, examples of microbiological wastes include specimen cultures, disposable culture dishes, discarded viruses, and devices used to transfer or mix cultures.
- **Pathological waste.** Unfixed human tissue (excluding skin), waste biopsy materials, and anatomical parts from medical procedures or autopsies.
- **Sharps waste.** Needles, glass slides and cover slips, scalpels, and IV tubing that have the needle attached.

There are 4 levels of biohazards, according to the Center for Disease Control:

- **Biohazard Level 1:** Agents that pose minimal threat to humans and the environment. Examples include *E. coli, Bacillus subtilis* and *Naegleria gruberi*.
- **Biohazard Level 2:** Agents that can cause severe illness in humans and are transmitted through direct contact with infected material. Examples include HIV, hepatitis B and salmonella.
- **Biohazard Level 3:** Pathogens that can become airborne and cause serious diseases. Examples include tuberculosis and *Coxiella burnetii*.
- **Biohazard Level 4:** Pathogens that pose a high risk of life-threatening disease for which there are no treatments. Examples include the Ebola virus and Lassa virus.

2. 

Blood collection tube is a sterile glass tube with a colored rubber stopper creating a vacuum seal inside of the tube facilitating the drawing of a predetermined volume of liquid. Vacutainer tubes may contain additives designed to stabilize and preserve the specimen prior to analytical testing. Tubes are available with a safety-engineered closure (rubber stopper), with a variety of labeling options and stopper colors as well as a range of draw volumes. Vacutainer tubes may contain additional substances that preserve blood for processing in a medical laboratory. Using the wrong tube may make the blood sample unusable for the intended purpose. These additives are typically thin film coatings applied using an ultrasonic nozzle.

The additives may include anticoagulants (EDTA, sodium citrate, heparin) or a gel with density between those of blood cells and blood plasma. Additionally, some tubes contain additives that preserve certain components of or substances within the blood, such as glucose. When a tube is centrifuged, the materials within are separated by density, with the blood cells sinking to the bottom and the plasma or serum accumulating at the top. Tubes containing gel can be easily handled and transported after centrifugation without the blood cells and serum mixing.

The meanings of the various colors are standardized across manufacturers.

The term *order of draw* refers to the sequence in which tubes should be filled. The needle which pierces the tubes can carry additives from one tube into the next, so the sequence is standardized so that any cross-contamination of additives will not affect laboratory results.

Containers containing coagulants

- Gold or "tiger" red/black top: clot activator and gel for serum separation
- Red top (plastic, not glass): clot activator but no serum separation gel
- Orange or grey/yellow "tiger" Top: thrombin, a rapid clot activator, for stat serum testing

Containers containing anticoagulants

- Green: sodium heparin or lithium heparin used for plasma determinations inclinical chemistry (e.g. urea and electrolyte determination). Sodium heparin collection tubes are the classically preferred tube for peripheral blood or bone marrow for cytogenetic studies. Lithium heparin is considered suboptimal for cytogenetics.
- Light green or green/gray "tiger": For plasma determinations.
- Purple or lavender: K_2 EDTA. This is a strong anticoagulant and these tubes are usually used for complete blood counts(CBC). Lavender top tubes are generally used when whole blood is needed for analysis. Can also be used for some blood bank procedures such as blood type and screen. EDTA tubes are preferred by most molecular genetics laboratories for molecular genetic studies (DNA or RNA).
- Grey: sodium fluoride and oxalate. Fluoride prevents enzymes in the blood from working, by preventing glycolysis so a substrate such as glucose will not be gradually used up during storage. Oxalate is an anticoagulant.
- Light blue: sodium citrate. Citrate is a reversible anticoagulant, and these tubes are used for coagulation assays.
- Dark Blue: EDTA.These tubes are used for trace metal analysis.
- Black - Used for Erythrocyte Sedimentation Rate (ESR).

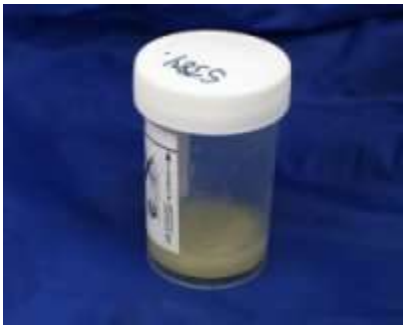
Other

- Red (glass): Contains no additives.
- Light yellow: Contains sodium polyanethol sulfonate (SPS). Used for blood culture specimens or acid-citrate-dextrose(ACD), used for blood bank studies, HLA phenotyping, and paternity testing.
- Tan (glass or plastic): Contains either sodium heparin (glass) or K_2EDTA (plastic). Used for lead determinations. These tubes are certified to contain no lead.

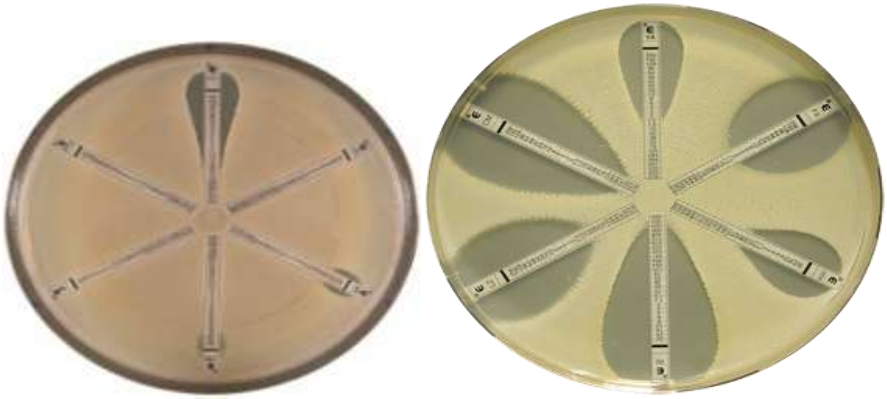
3.  sample containers

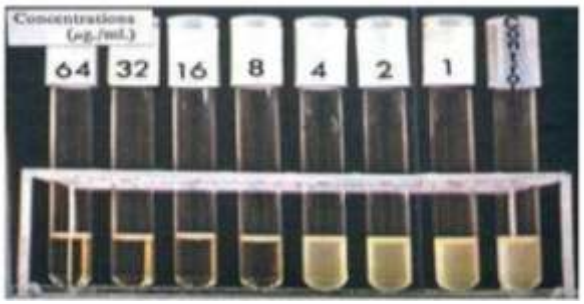
4.  sample container with stool sample

5.  Sample container with urine sample

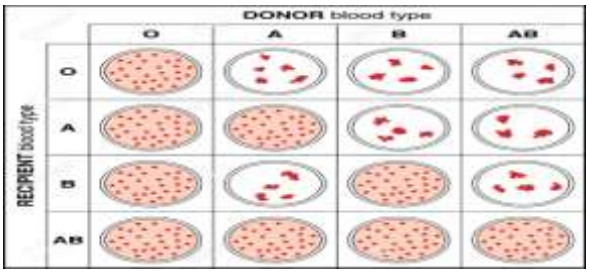
6.  Sample container with Sputum sample

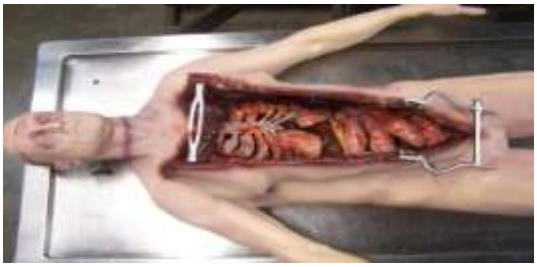
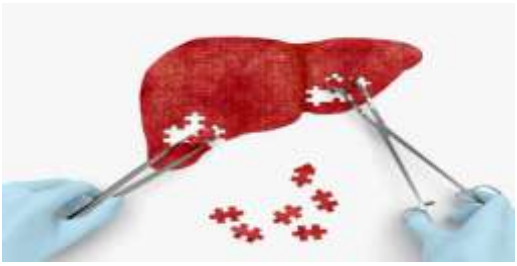
7.  Antibiotic Susceptibility test

8.  MIC – E test

9. 

MIC

10. Differential media - MacConkey agar with lactose fermenting colonies
11. Selective media – EMB agar with Metallic Sheen colonies
12. Selective media – cetrimide agar with green colonies
13. Selective media – TCBS agar with yellow colonies
14. Selective media – SS agar with black colonies
15. Selective media – XLD agar with black colonies
16. Basal media – Nutrient agar
17. Basal media – Nutrient agar with quadrant streaking
18. Inoculation loop
19. Sterile swabs
20. Antibiotic discs, powders
21. Sterile discs
22. Widal kit
23. Blood grouping kit 

24. Autopsy and biopsy  
25. Gloves, aprons, masks