Experiment 1

Aim: Study of permanent slides and specimens of parasitic protozoans and helminthes.

Background: The taxonomy and classification of parasitic protozoans and helminths includes knowing the different classes, orders, and families to which these organisms belong, investigation of the morphological features of both protozoans and helminths and focus on characteristics such as size, shape, colour, and specialized structures (e.g., hooks, suckers, flagella) that are unique to each species. It helps to explore the life cycles of the parasitic organisms and understand the various stages (egg, larva, adult) and the host organisms involved. It helps to connect the morphological features and life cycles of parasitic protozoans and helminths to the clinical manifestations of the diseases they cause in humans or animals. Such type of studies also involves to examine the interactions between parasites and their hosts and to understand how parasites establish infection, evade host defences, and cause disease. This includes exploring the pathological changes induced by these parasites in host tissues. To consider the epidemiological aspects of parasitic infections and to understand the distribution of these parasites, risk factors for transmission, and public health implications. This knowledge is essential for designing effective control and prevention strategies.

Procedure

1. Preparation the microscope: Ensure the microscope is clean and in good working condition. Turn on the light source and adjust the intensity to a comfortable level. Set up the microscope slide holder or stage and secure the slide in place.
2. Preparation the specimen: If using a pre-prepared permanent slide, ensure it is clean and free from debris. If you have prepared your own slide, ensure the specimen is properly mounted and covered with a cover slip.
3. Cleaning of microscope lenses: Use lens cleaning paper or a lens cleaning cloth to clean the objective and eyepiece lenses. Any dirt or fingerprints on the lenses can affect the clarity of the image.
4. Focus of the microscope: Use the coarse adjustment knob to bring the specimen into rough focus. Fine-tune the focus using the fine adjustment knob for a clearer image.
5. Magnification: Once you have a clear view at low magnification, increase the magnification by rotating the nosepiece to the next higher objective (e.g., 40x). Refocus using the fine adjustment knob. Adjust the lighting as needed to enhance

contrast and clarity. This may involve adjusting the diaphragm or using additional features on the microscope.

6. Observation and record: Carefully observe the specimen. Take note of its morphology, structures, and any relevant details. If needed, use a microscope camera or smartphone attachment to capture images.
7. Cleaning: After observation, lower the stage and rotate the nosepiece to the lowest magnification objective. Power off the microscope and clean the lenses again.

Experiment 2

Aim: Pathological examination of sputum, blood, urine and stool.

Background: Examination of sputum, blood, and urine is crucial in medicine for diagnostic purposes, monitoring health conditions, and guiding appropriate treatment. For sputum, examination is necessary because of the identification of pathogens such as bacteria, viruses, fungi, and parasites during respiratory infections. It includes detection of *Mycobacterium tuberculosis* using staining techniques (e.g., Ziehl-Neelsen stain) for tuberculosis screening, evaluation of cellular components to assess inflammation, presence of blood, or abnormal cells for a good respiratory health. Such studies helps in selecting appropriate antibiotics or antifungal medications based on the identified pathogens.

Pathological examination of blood includes identification of various diseases, including infections, anaemia, leukaemia, and metabolic disorders. Continuous monitoring of complete blood count (CBC) provides insights into the overall health of the individual. Blood chemistry tests assess the functioning of organs, including the liver, kidneys, and pancreas. Blood cultures help identify systemic infections and guide appropriate antimicrobial therapy.

Urine examination helps in renal function assessment by identification of kidney disorders, urinary tract infections, and metabolic abnormalities. Presence of glucose in urine may indicate diabetes. Presence of hCG (human chorionic gonadotropin) in urine is used for pregnancy testing. Some drugs and their metabolites can be detected in urine, aiding in substance abuse screening.

Stool provides identification of pathogens causing gastrointestinal infections, including bacteria, parasites, and viruses. Examination of stool helps in identifying parasites and their ova. Detection of occult blood in stool may indicate gastrointestinal bleeding. Observation of stool characteristics aids in assessing digestive health and function.

Procedure

1. For pathological examination of sputum
    a. Patient preparation: Instruct the patient on the proper method of sputum collection. Advise the patient to rinse the mouth with water before collecting the sputum to minimize contamination.

b. Sputum collection: Ask the patient to take a deep breath and then cough forcefully to produce sputum. Collect the sputum directly into the sterile sputum container. Ensure that the specimen is from the lower respiratory tract and not saliva.
c. Labelling and identification: Label the sputum container with the patient's name, identification number, date, and time of collection. Clearly specify any specific tests requested on the request form.
d. Microscopic examination: Macroscopic examination for consistency, color, and presence of blood. Microscopic examination for the presence of cells, microorganisms, and other abnormal elements. Culture and sensitivity testing to identify any bacterial or fungal pathogens and determine their susceptibility to antibiotics.
e. Cytological examination: For suspected malignancies, a cytological examination may be performed on the sputum specimen to detect abnormal cells. This may involve staining and microscopic evaluation by a pathologist.

2. For pathological examination of blood
   a. Patient identification and consent: Verify patient identity using at least two identifiers. Explain the procedure to the patient, addressing any concerns and obtaining informed consent.
   b. Preparation of collection site: Choose an appropriate site for blood collection (usually the antecubital vein). Disinfect the skin using an alcohol swab or antiseptic wipe.
   c. Blood collection: Put on gloves and other required PPE. Collect blood using a sterile needle and syringe or a vacuum tube system. Fill the appropriate blood collection tubes with the necessary anticoagulants or additives for specific tests (e.g., EDTA for CBC)
   d. Laboratory procedure: in the laboratory the blood under goes different procedures like complete blood count (CBC), differential blood count and blood smear for other analysis of morphology and abnormalities in the blood cells.

3. For pathological examination of Urine
   a. Patient preparation: Instruct the patient on the proper method of urine collection. Advise the patient to cleanse the genital area before urine collection.

b. Collection of urine: Use a sterile urine collection container. Collect a midstream portion of urine to minimize contamination from the initial and final portions of the stream.
c. Physical examination: visual inspection by checking the colour, quality and odour of the urine. A refractometer may be used to measure the concentration of solutes in the urine.
d. Chemical analysis: Use urine dipstick tests for the semi-quantitative analysis of various chemical components like pH, glucose, protein, ketones, bilirubin, urobilinogen, nitrites, leukocytes, and blood.
e. Microscopic examination: Centrifuge a portion of the urine to concentrate the sediment. Examine the sediment under a microscope for the presence of cells, crystals, casts, bacteria, and other particles.
f. Pathological test: Pathologists and laboratory professionals generate a detailed report based on the findings from the urine examination. The report is sent to the requesting healthcare provider, contributing to the overall diagnostic process.

4. For pathological examination of stool
   a. Patient preparation: Instruct the patient on the proper method of stool collection. Advise the patient to avoid urine contamination during collection. Use a sterile stool collection container. Collect a sample from different parts of the stool to ensure a representative specimen.
   b. Physical examination: visual inspection for colour, consistency, and the presence of blood or mucus. Note any unusual or foul odours.
   c. Microscopic assessment: Prepare a stool smear on a microscope slide. Examine the smear for the presence of *Giardia lamblia*, *Cryptosporidium*, or *helminth* eggs as parasite. Including *Clostridium difficile* or pathogenic strains of bacteria. WBC for inflammation, RBC for gastrointestinal bleeding, Fat globules indicating malabsorption.
   d. Culture: In cases of suspected bacterial infections, culture the stool on appropriate selective media. Incubate the culture for the specified duration to allow the growth of bacteria.
   e. Pathological laboratory: for detailed analysis send the sample to pathologist.

Experiment 3

Aim: Blood: Erythrocyte Sedimentation Rate (ESR), Haematocrit.

Background: The test is based on the principle that during inflammation, certain proteins, particularly fibrinogen, increase in the blood. These proteins cause red blood cells to aggregate and form stacks or "rouleaux." The rouleaux settle more rapidly than individual red blood cells, leading to an increased sedimentation rate. Blood is composed of cellular elements (red and white blood cells) and plasma. Haematocrit is the ratio of the volume of red blood cells to the total blood volume. Anaemia, characterized by a decrease in red blood cells, will result in a lower haematocrit, while conditions such as dehydration or polycythaemia can lead to elevated levels. Elevated ESR is a non-specific indicator of inflammation and can be seen in various conditions, including infections, autoimmune diseases, and certain cancers. Normal reference ranges may vary with age and sex. For male $\leq$ 15 mm/hr, female $\leq$ 20 mm/hr and for child $\leq$ 10 mm/hr. Low haematocrit values may indicate anaemia, nutritional deficiencies, or chronic diseases affecting red blood cell production. High haematocrit values may suggest dehydration, lung or heart diseases, or conditions like polycythaemia vera. Normal haematocrit value for males is 38.8%-50.0% and in females 34.9%-44.5%.

Procedure

1. For ESR examination: -
    a. Blood is mixed with an anticoagulant to prevent clotting.
    b. The blood is placed in a vertical tube, and the rate at which red blood cells settle is measured over a specific time period, usually one hour
    c. The result is reported as the distance (in millimeters) that the red blood cells have descended in the tube during this time
    d. Formula used- ESR (mm/hr) = distance red blood cells fall/time in hours.
2. For Haematocrit examination: -
    a. Blood is collected into a capillary tube and then centrifuged.
    b. The centrifuge separates blood components based on their density, causing red blood cells to settle at the bottom.
    c. The haematocrit is calculated by measuring the percentage of the total tube length occupied by the red blood cell layer.

d. Formula used- haematocrit (%) = (volume of red blood cells/total blood volume) X 100

Experiment 4

Aim: Staining and identification of Gram positive and Gram-negative bacteria.

Background: The Gram staining technique is a widely used method in microbiology for the differentiation of bacteria into two main groups: Gram-positive and Gram-negative. Developed by Hans Christian Gram in the 1880s, this staining method is fundamental in bacterial identification and classification. The classification into Gram-positive or Gram-negative is based on the reaction of the bacterial cell wall to the decolorization step. Gram staining is a rapid and essential tool in microbiology, aiding in bacterial identification and guiding further diagnostic tests. Helps in the diagnosis of bacterial infections and guides antibiotic therapy.

Procedure

1. Preparation of bacterial smear: Aseptically transfer a small amount of bacterial culture to a clean microscope slide. Spread the bacterial material in a thin, even layer on the slide. Allow the smear to air dry or heat fix it by passing the slide through a flame a few times.
2. Primary staining: Flood the heat-fixed bacterial smear with crystal violet stain for 1 minute. Rinse the slide gently with water to remove excess crystal violet.
3. Mordant addition: Apply Gram's iodine solution (iodine in potassium iodide) to the stained smear. Allow the iodine to act as a mordant for 1 minute. Rinse the slide gently with water to remove excess iodine.
4. Decolorization: Apply ethanol or acetone drop by drop until no more colour is being washed off. This step differentiates between Gram-positive and Gram-negative bacteria. Rinse the slide quickly with water to stop the decolorization process.
5. Counterstaining: Flood the smear with safranin counterstain for 1-2 minutes. Rinse the slide gently with water to remove excess safranin.
6. Drying and mounting: Allow the slide to air dry. Optionally, a coverslip can be placed over the stained smear using a mounting medium.
7. Microscopic examination: Examine the stained bacterial smear under a light microscope. Gram- Positive Bacteria will retain the crystal violet stain and appear purple/blue. Gram- Negative Bacteria will take up the safranin counterstain and appear pink/red.

Experiment 5

Aim: Demonstration of antigen-antibody interaction in gel.

Background: Immunodiffusion is a laboratory technique used to study the interaction between antigens and antibodies. Developed by Ouchterlony in the mid-20th century, this method exploits the principle that when antigens and antibodies diffuse toward each other through a gel medium, they form visible precipitin lines where they meet. This technique is fundamental in immunology and has various applications, including antigen quantification and the identification of specific antibodies.

Procedure

1. Preparation of Agarose Gel: Prepare and pour a suitable concentration of agarose gel into a Petri dish. Allow the gel to solidify.
2. Well preparation: Using a suitable tool, make wells in the solidified agarose gel. These wells will hold the antigen and antibody solutions.
3. Adding of antigen and antibody: In separate wells, carefully add the antigen solution and antibody solution. Ensure that the solutions do not mix prematurely.
4. Incubation: Allow the antigen and antibody to diffuse through the gel. Incubate the gel at an appropriate temperature, allowing sufficient time for diffusion and interaction to occur.
5. Observe precipitin lines: After incubation, observe the gel for the formation of precipitin lines, which indicate the interaction between antigens and antibodies. These lines are formed at the point where antigen and antibody concentrations are optimal for complex formation.
6. Measure precipitin lines: Measure the diameter of the formed precipitin lines as an indicator of the strength of the antigen-antibody interaction.
7. Control experiments: antigen control, antibody control, double diffusion control.
8. Staining (optional): Optionally, stain the gel using suitable staining techniques to enhance visualization of precipitin lines.
9. Document the results, including the presence and characteristics of precipitin lines.

Experiment 6

Aim: Separation of γ-globulin by salt precipitation.

Background: The separation of γ-globulin by salt precipitation is a technique commonly used in protein biochemistry and immunology. This method exploits the solubility characteristics of proteins in response to changes in salt concentration. The primary principle behind this separation is the selective precipitation of γ-globulins (immunoglobulins) from a mixture of proteins by adjusting the salt concentration. γ-globulins, or immunoglobulins, are a class of proteins involved in the immune response. They have unique solubility characteristics that make them amenable to salt precipitation. The salt concentration in the solution is carefully adjusted to a level that selectively precipitates γ-globulins while leaving other proteins in solution. γ-globulins form insoluble aggregates or precipitates in response to the increased salt concentration. The precipitate can be collected by centrifugation or filtration. This technique is commonly used in the purification of specific classes of immunoglobulins, including γ-globulins. Isolated γ-globulins can be used in diagnostic assays, such as immunodiffusion or immune- electrophoresis.

Procedure

1. Prepare γ- globulin- containing solution: Start with a solution containing γ-globulins. This can be a serum sample or a purified γ-globulin solution.
2. Buffer adjustment: Add a suitable buffer to the solution to maintain the desired pH. The pH should be within a range that ensures stability and solubility of γ-globulins.
3. Gradual addition of salt: Begin the salt precipitation by gradually adding the selected salt (e.g., ammonium sulfate) to the solution with stirring. Add the salt slowly to avoid sudden changes in protein environment and precipitation. Stir continuously during the addition.
4. Monitor salt concentration: Measure the salt concentration in the solution using a salt concentration meter or by monitoring the weight of salt added.
5. Continue stirring: Continue stirring for an additional period to allow for complete precipitation of γ-globulins.
6. Centrifugation: After the completion of the precipitation process, centrifuge the solution at a suitable speed and duration. Centrifugation separates the precipitated γ-globulins from the remaining solution.

7. Decant supernatant: Carefully decant the supernatant, leaving the precipitated γ-globulins in the bottom of the tube.
8. Redissolution: Redissolve the precipitated γ-globulins in a suitable buffer for further analysis or use. Phosphate-buffered saline (PBS) is commonly used for redissolution.
9. Analysis: Analyse the separated γ-globulin fraction for purity and concentration using techniques such as gel electrophoresis, immune electrophoresis, or enzyme-linked immunosorbent assay (ELISA).

Experiment 7

Aim: Preparation of thin and thick blood films to diagnose Plasmodium infections.

Background: Thick and thin blood smear study is the gold standard method for malaria diagnosis. The procedure follows these steps: collection of peripheral blood, staining of smear with Giemsa stain and examination of red blood cells for malaria parasites under the microscope.

Thick smear: It is not fixed in methanol; this allows the red blood cells to be hemolyzed, and leukocytes and any malaria parasites present will be the only detectable elements. However, the hemolysis may lead to distorted plasmodial morphology making plasmodium species differentiation difficult. Therefore, thick smears are mainly used to detect infection and to estimate parasitemia.

Thin smear: It is fixed in methanol. Thin smears allow the examiner to identify malaria species, quantify parasitemia, and recognize parasite forms like schizonts and gametocytes.

Procedure:

a. For thick smear:

1. Clean the slide and ring finger of person with ethanol.
2. Prick the finger with sterilize needle and place 2 – 3 drops of blood (approx. 5µL)on the clean slide.
3. Spread the drop rapidly with the corner of another slide to form a circular area measuring approximately 1.5 – 2cm in diameter.
4. Leave the film to dry at room temperature for 15 minutes.
5. Place the film into a 37˚C incubator for 5 minutes.
6. Stain the slide with 3% Giemsa stain for 45 – 60 minutes (exposure time determined according to site environment))
7. Pour of the remaining stain and rinse with bufered water.
8. Now slide is allowed to air dry and then mount it with DPX mountant and put cover slip on it for longevity of samples
9. Observe the slide in 100x under light microscope.

b. For thin smear:

1. Clean the slide and ring finger of person with ethanol.
2. Prick the finger with sterilize needle and place a small drop (between 2-5ul depending on viscosity of sample) at one end of a clean slide.
3. Using a spreader or another slide, at an angle of approximately 45 degrees, bring the spreading slide backwards into the drop of blood, allowing it to spread along the edge.
4. Push the spreader forward in a steady movement to produce a film that has two straight edges with a feathered 'tail' that does not reach the end of the slide.
5. Allow the film to dry at room temperature.
6. Fix the thin film using methanol and allow the methanol-fixed thin smear to dry completely in air (around 2 minutes).
7. Pour of the remaining stain and rinse with bufered water.

8. Now slide is allowed to air dry and then mount it with DPX mountant and put cover slip on it for longevity of samples
9. Observe the slide in 100x under light microscope.

Experiment 8

Aim: Preparation of temporary and permanent slides of faecal matter by saline preparation and concentration techniques to identify cysts of parasitic protozoans and helminthes eggs.

Background: Stool wet mount preparation is the simplest and basic method of analyzing a stool specimen in coprology. It utilizes a saline solution as an isotonic media to maintain the cellular structure of the various pathogens (like protozoan trophozoites, cysts, oocysts, and helminth eggs and larvae) that are found in stool and that we like to examine. The motile stage of protozoan parasites were identified using direct wet mount.

Stool concentration method is used when parasite number is low. **Following method**s **used for the concentration of stool:**

1. **Formalin-ethyl-acetate concentration method:** It is most commonly used method. This method recovers the helminth eggs and larvae, to a lesser extent, trophozoites. This is based on specific gravity. After centrifugation, the stool's parasites are heavier and settle at the bottom as sediments.
2. **Zinc-floatation method:** The parasites are lighter and float on the surface, while the debris settles at the bottom.

Procedure:

- **Stool wet mount technique:**

1. Clean a slide using ethanol.
2. Put the fresh stool sample (2 mg of stool) on a slide with a wooden applicator stick.
3. The stool is emulsified with a drop of physiological saline (0.85%) for diarrheic and semi-solid samples. (For formed stools, iodine is used)
4. Then, put the cover slip on it and examine it under a microscope using first 10x objectives and then 40x objectives.

- **Stool concentration method:**

Formalin-ethyl-acetate concentration method:

1. Mix 2 to 5 grams of the stool thoroughly in the 10% formalin. The stool should be fixed in formalin for at least 30 minutes.
2. Filter the above stool in the formalin. This can be done by two layers of gauze or a wire screen and collecting around 3 mL.
3. Add 10 to 12 mL of 0.85% saline and mix it well.
4. Centrifuge for 2 minutes at 2000 RPM (or 2500 RPM).
5. Discard the supernatant and leave 1 to 1.5 mL of the sediment. (If the supernatant is cloudy, then repeat the above steps of saline)
6. Add 9 mL of 10% formalin to the sediment.
7. Now add 3 mL of ethyl acetate.
8. Cap the test tube and shake well for 30 seconds.
9. Centrifuge the tubes for 1 minute at 2000 RPM.
10. Four layers will form. The bottom is the sediment that is needed to prepare the smear.
11. Remove the debris with a wooden applicator stick. Decant the upper three layers carefully and leave the sediments in the test tube.
12. Clean the sides of the test tube with a swab. (Giardia cyst may stick to the side of the test tube)

13. Add a few drops of the formalin and mix the sediment thoroughly. This will preserve the sediment.
14. Now, we can make the smears in saline and iodine wet preparation.
15. Examine under the microscope.

Zinc-floatation method:

1. Fix the stool in the formalin.
2. Make a dilution of the above specimen (1 mL) with tap water from 1:10 to 15.
3. Pour the above suspension through a funnel with two layers of gauze in a small test tube.
4. Add 2 mL of ether to the test tube with a stopper and gently shake.
5. Now add water to the above test tube to the top just 1 cm from above.
6. Centrifuge at 2500 rpm for 45 seconds.
7. Decant the supernatant.
8. Add 2.5 ml of the water to the sediment, shake well to resuspend the sediment—repeat steps 5 and 6.
9. Add 2.5 ml of zinc sulfate ($ZnSO_4$) to the sediment to resuspend it.
10. Add zinc sulfate solution to the top of the test tube, leaving only 1 to 0.5 cm open on top.
11. Centrifuge the test tube for 2 minutes at 2000 RPM.
12. Take the surface material with a wire loop; this is the material where you can see the parasites.
13. Make wet preparation with saline and iodine.
14. Examine under the microscope.

For permanent slides of faecal matter: (SAF Procedure)

1. Prior to use, make a working solution by mixing equal parts of the Iron-hematoxylin mordant and the stain (This working solution should be prepared fresh weekly)
2. Prepare a smear by mixing 1 drop of Mayer's albumin with sediment from the SAF preserved specimen.
3. Allow slide to air dry at room temperature until smear is dry and opaque.
4. Place slide in 70% alcohol for 5 minutes.
5. Wash in container of tap water for 2 minutes.
6. Place slide in Kinyoun stain for 5 minutes.
7. Wash slide in running tap water for 1 minute.
8. Place slide in Kinyoun decolorizer for 4 minutes.
9. Wash slide in running tap water for 1 minute.
10. Place slide in Iron Hematoxylin working solution for 8 minutes.
11. Wash slide in distilled or deionized water in container for 1 minute.
12. Place slide in picric acid working solution for 3 to 5 minutes.
13. Wash slide in running tap water for 10 minutes.
14. Place slide in 70% alcohol plus ammonia for 3 minutes.
15. Place slide in 95% ethyl alcohol for 5 minutes.
16. Place slide in 100% ethyl alcohol for 5 minutes.
17. Place slide in two changes xylene or xylene substitute for 5 minutes.

18. Add permount to the stained area and cover with a coverslip.
19. Examine slides microscopically with the 100X objective