HAEMATOLOGY AND BLOOD TRANSFUSION

MLS 2207/2208: CLINICAL HAEMATOLOGY

OSMOTIC FRAGILITY TEST

This is a hemolytic screening test for red cell volume, surface area and membrane defects.

PRINCIPLE

Red cells will haemolyse when subjected to hypotonic solution of NaCl and the degree of lysis is read colorimetrically.

Water moves from a low concentration to high concentration. This happens because the osmotic pressure is high in higher concentration than in low.

MATERIALS REQUIRED

1. Stock saline solution1% (prepare serial dilutions 0.1, 0.2, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.65, 0.75 & 0.85%)
2. Tests tubes
3. 0.5ml pipette
4. Colorimeter
5. Incubator
6. Filter 540nm
7. Centrifuge
8. Specimen- Heparinised (Not EDTA because it is a salt and if used it will increase NaCl concentration)

Procedure

1. Arrange 12 tubes in a row and label them accordingly; 0.1,0.2…….0.85%
2. Pipette 5ml of each dilution of NaCl solution in each accordingly.
3. Add 0.05ml of well mixed blood into each tube.
4. Mix and stand for 30mins at room temperature.
5. Centrifuge the contents of all the tubes at 1500RPM for 5mins to separate the haemolysate from the stroma.
6. Read the OD of the supernatant of all tubes at 540nm.
7. Convert the OD to % haemolysis using the formula.

100% haemolysis= O.D 0.1%dilution

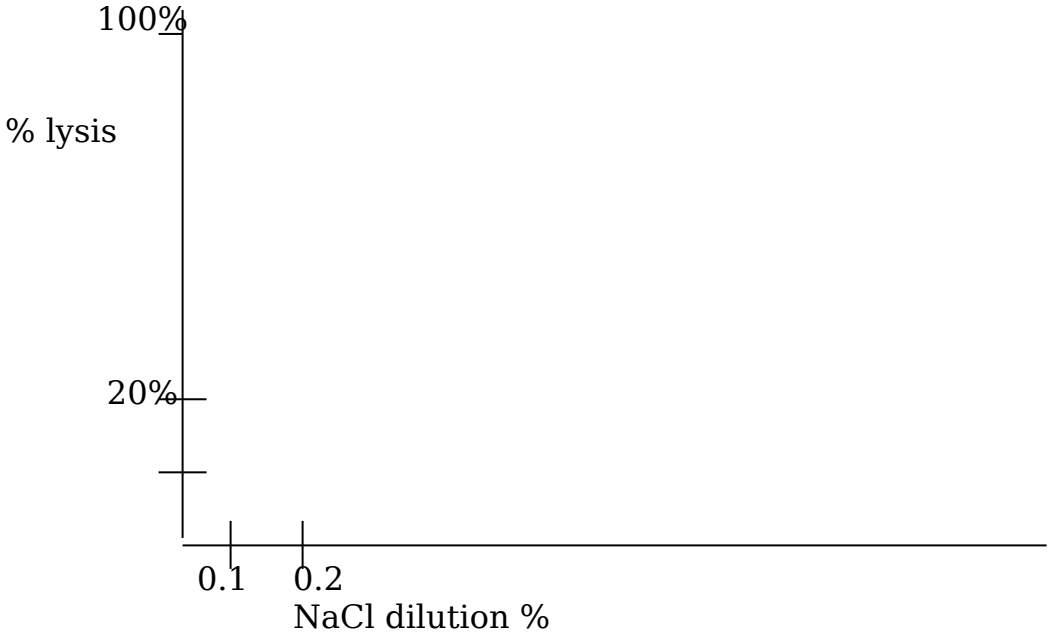
$$\frac{\text{O.D of test}}{\text{O.D of STD}} \times 100$$

MCOF= Mean cell Osmotic Fragility

Normal range= 0.4--0.45
NaCl concentration giving us 50% lysis is referred to as MCOF which is normally 0.4 –0.45% dilution.

8. Plot a graph of % lysis against NaCl concentration.

From that graph establish the mean cell osmotic fragility (MCOF) which is the NaCl concentration where there was 50% lysis.



e.g.

NaCl dilution %	O.D	%lysis
0.1	0.6	100%
0.2	0.5	83%
0.3	0.45	75%
0.4	0.4	67%

Using the formula O.D of test/O.D of STD X 100 and use 0.1% dilution as the standard.

Questions

1. What conditions lead to low osmotic fragility
2. What conditions lead to high increased O.F.?
3. What are the advantages of using heparin anticoagulant for sample collection in this test?

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ANTIBODY TITRATION

Requirements;

Pipettes, test-tubes, agglutination tubes, grease pencil, racks, serum saline, antigen and 50C water bath.

Method;

1. Place ten (10) test tubes in a rack.
2. Add 4ml of saline to tube 1 and 1ml to tube 2-10.
3. Add 1ml of serum to tube 1 and mix. This dilute serum 1 in 5 i.e. 1 vol. of serum plus 4 vol. of saline.
4. Transfer 1ml of serum saline from tube 1 to tube 2. This dilutes serum in tube 2 into 1 in 10.
5. Repeat the procedure up to and including tube 10. This gives serum dilutions of 1in 5, 1 in 10, 1 in 20, 1 in 40, 1 in 80,1 in 160, 1 in 320, 1 in 640, 1 in 1280 and 1 in 2560.
6. Using fresh pipette and starting from the highest dilution, transfer 0.5ml from each test tube into corresponding agglutination tube rack.
7. Add 0.5ml of antigen to each tube. The addition of equal 1 vol. to antigens dilutes the serum again. The final serum dilutes being 1 in 10 in tube1, 1 in 20 in tube 2 and so on.
8. To another agglutination tube add 0.5ml of saline and 0.5ml antigen. This tube serves as control and shows if antigen is salt-agglutinable.
9. Place the rack in the water bathe and adjust water level to cover one third of the tube. This sets up convection currents which assist in keeping the serum/antigen suspension mixed.
10. Incubate at 50C and examine at 1, 4, and 24hrs intervals.

N/B:

The highest serum dilution at which agglutination occurs is noted and this dilution denotes the titre of the serum.
The greater the concentration of antibodies present the higher the titre becomes.

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BLOOD COLLECTION

Blood must be collected with care and adequate safety precautions to ensure reliable results. Avoid contamination and infection from the blood transmissible pathogens. Wear protective gloves when collecting and handling blood samples, use dry sterile needles, lancets and syringes. Discard blood collecting materials safely to avoid injury from needles and lancets.

Areas of collecting Blood,

Capillary – Finger, Heel of an infant, Ear lobe
Venous – Arm vein, Jugular vein, Femeral vein

CAPILLARY

Mainly used when the patient is an infant or young child and the volumes of blood required is small, e.g. to measure HB, perform WBC count, making thin & thick film.

Disadvantages

- Only used for a few tests.
- Greater chances of sampling errors particularly when blood is not free flowing.
- Difficulty in obtaining sufficient blood particularly when required for more than one test
- Test cannot be repeated immediately or further tests done when results are unexpected or abnormal.

Capillary blood can be collected from

- The ‘ring’ finger of a child or adult (do not stick the thumb or index finger as these are most sensitive)
- The heal of an infant up to 1year old. (take care not to damage the heal by sticking too near the edge)

Technique

N/B

Make sure the area is warm to allow free flow of blood, on cold days soak the hand or foot of an infant in warm water prior.

- Cleanse the puncture area with 70% alcohol and allow to dry.
- Using a sterile pricker, needle or lancet, make a rapid, sufficiently deep puncture to allow free flow of blood.
- Wipe away the first drop of blood with a dry cotton wool & use the next few drop.

- When sufficient blood has been collected, press apiece of dry cotton wool over the area until bleeding stops.

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VENOUS BLOOD

Anticoagulated blood is used when more than 100ul of blood is required or when serum is required, e.g. in compatibility tests or antibody tests. Venous is preferred to capillary particularly when the patient is an adult & tests required are several.
Various anticoagulants are used for various tests, e.g. EDTA, Heparin, Sodium fluoride, Potassium Oxalate, Sodium citrate.

Technique

Staff must NOT collect blood unless they have been adequately trained and newly qualified staff MUST be supervised until they have gained sufficient experience. When venous blood is required from an infant this should be done by a medical officer or an experienced Phlebotomist.

- Select a sterile, dry, preferably plastic syringe of the capacity required e.g. 2.5ml, 5ml or 10ml attach it to a needle (vacuteiner needle and a holder may also be used)
- Reassure the patient on the procedure to be undertaken, if the patient is a minor explain to the guardian.
- Apply a soft tubing tourniquet or Velcro fastening arm band to the upper arm of the patient to enable visibility of the vein. (do not tie too tightly or for too long than 2minutes) Ask the patient to make a fist to make the vein more prominent.
- Using the index finger select a suitable vein that is large straight and does not roll.
- Cleanse the puncture site with 70% ethanol and allow to dry. Do not re-touch the cleansed area.
- With the thumb of the left hand holding down below the puncture site, make the venipuncture with the bevel of the needle directly upwards in the line of the vein. Steadily withdraw the plunger of the syringe at the speed it is taking the vein to fill. Avoid moving the needle in the vein.
- When enough blood has been collected release the tourniquet & instruct the patient to release the fist. Remove the needle and immediately press on the site with a dry cotton wool, instruct the patient to hold the cotton wool until bleeding has completely stopped.

- Carefully remove the needle from the syringe and discard it safely, carefully fill the container(s)

N/B

Do not fill the container with the needle attached to the syringe as this causes haemolysis. Do not try to re-sheath (cork) as this may result to a needle stick injury.

- Mix the blood well with the anticoagulant, label the container with the patient laboratory number.

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ESR-(ERYTHROCYTE SEDIMENTATION RATE)

Requirements:

1. Wintrobe tubes, Westergreen tubes
2. 3.8% sodium citrate, Anticoagulated blood
3. Stands/ Racks, Long pastuer pipette

N/B; 3.8% sodium citrate is 3.8gms of sodium citrate in 100mls of distilled water.

Principle

When a column of anticoagulated blood is left standing vertically for an hour, erythrocytes fall through the plasma and settles at the bottom by sheer gravitational force. The distance moved by the cells is the ESR and is measured in millimeters per hour.
This process is in three stages: Aggregation, maximum fall and packing.

Method:

Various methods are involved; Wintrobe, Westergreen, Landau-Adams and Smith's.
Landau-Adam's and Smith's are micro-methods used for small specimens.

WINTROBE

EDTA or Oxalate anticoagulated blood is used.

(i) Well mixed anticoagulated venous blood is delivered into the tube up to zero mark using a pastuer pipette.

(ii) The tube is then placed in a rack/ standing a vertical position and left to stand for 1hour at room temperature.

(iii) The distance moved by the cells after an hour is read and recorded as the ESR in mm/hr.

Reference values: Male- 4-9mm/hr, Female- 5-15mm/hr

WESTERGREN

3.8%Sodium citrate anticoagulated blood is used. (9volume of blood in 1volume of citrate)

(i) Well mixed anticoagulated blood is put into the tube upto zero mark using a pastuer pipette.

(ii) The be is then clamped in a vertical position on a stand/rack and left to stand

(iii) The distance moved by the red cells from the zero mark is read and recorded as the ESR in mm/hr.

MICRO-ESR

It is like westerngreen ESR except that capillary blood is used. The blood may be taken from the heal, earlobe or finger prick. It is mainly done to 1-2year babies.

(i) Warm the area to be pricked then mark the tube at a volume of 1.25mls. Add 0.25mls of sodium citrate at a concentration of 5%.

(ii) Sterilize the area with 70%alcohol or methylated spirit, make a clean deep prick on the sterile area and draw blood straight into the citrated tube mixing constantly.

(iii) When the 1.25ml mark is attained then carryout a westergreen test as usual.

QUESTIONS: **1.** what are the advantages and disadvantages of each method?

2. Write sources of error.
3. What is the importance of the test?
4. Write causes of high ESR.
5. Write causes of low ESR.

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SICKLING TEST

Requirements:

(a) 2% sodium metabisulphate solution. (dissolve 2gms of sodium metabisulphate in 100ml of Distilled water)
(b) Glass slides.
(c) Coverslips.
(d) Candle wax or Vaseline jelly.

Principle:

Under anoxic environment, erythrocytes that contain HBS be it in Sickle cell anaemia, Sickle cell trait or other sickle cell disease states become sickled. This process is hastened by incubating the HBS containing erythrocyte with a potent reducing agent such as sodium metabisulphate at 370C

Procedure:

(i) Place a small drop of blood (capillary or venous) at the centre of a clean glass slide.

(ii) Add a drop of 2% freshly prepared sodium metabisulphate and mix well using an applicator stick.
(iii) Place a cover slip over the preparation and seal the edges with petroleum jelly or paraffin wax avoiding air bubbles.
(iv) Incubate for 2hours at 370C then examine at power X40 objective for sickle cells.

If negative, incubate overnight and re-examine.

Results:
Sickle cells present- POSITIVE
Sickle cells absent- NEGATIVE.

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HAEMOGLOBIN SOLUBILITY TEST (S-TEST)

The technique is useful to field workers who may not have incubation facilities for sickling test or electrophoresis. It is quick for screening purposes, cheap and reliable. The results are available within 5 minutes.

REQUIRMENTS:
(i) Whole blood with Hb AA, S and Ss.
(ii) 75x10mm clean test tubes.
(iii) 2ml and 0.02ml pipettes.
(iv) Reagents; Mixture of Sorensens buffer.
Sodium clithionite and saponin.

METHOD:

(i) Take 2ml of the mixture into 3 tubes labeled AA, Ss and TT (test).

(ii) Add 0.01ml of whole blood to respective tubes. Mix and leave at room temperature for 5minutes.

(iii) After 5minutes take a piece of paper with print and hold 1cm behind each tube and read the letters through the solution in each tube.

RESULTS:

Tube AA should be clear and letters read through.

Tube AS and Ss show turbidity and letters invisible if one tries to read through.

CAUTION:

Addition of saponin is critical. Too much will be falsely even HbS and the solution will be falsely clear.

Too little saponin will on the other hand fail to dissolve red cell membranes in Hb and turbidity will appear indicating false positive HbS present.

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RETICULOCYTES COUNT.

MATERIALS (REQUIRMENTS)
New Methylene blue or brilliant cresyl blue

Preparation:
1.0g brilliant cresyl blue dissolved in 100ml of citrate-saline solution. (1part of 3%sodium citrate to 4parts of 0.9%sodium chloride) Filter the mixture and its ready for use.

- Test tubes, Pasteur pipettes
- Glass slides, Tally counter
- Water bath, Microscope

PRINCIPLE
Reticulocytes are juvenile red cells; they contain remnants of the basophilic rebonucleoprotein (reticulum) which was present in large amounts in the

cytoplasm of the nucleated precursor. This basophilic material has the property of reacting with certain dyes such as brilliant cresyl blue or new Methylene blue to form a blue precipitate of granules or filaments. This reaction takes place only intravitally stained, unfixed preparations. The number of reticulocytes in the peripheral blood is a fairly accurate reflection of erythropoietic activity of the marrow.

METHOD

- 2-3 drops of staining solution, new methylene blue or cresyl blue are put into a 72x10mm test tube by means of Pasteur pipette.
- Equal volumes of anticoagulated blood are added to the dye solution.
- Mix and incubate at 37^{o}C for 15-20 minutes.
- Resuspend the mixture by gently mixing and make thin smears on a clean glass slide and allow to them to dry.
- Examine the smears when dry using the oil immersion objective and/or erlichs eye-piece if available.

NB; A piece of paper with w square hole at the center, placed on the eye-piece serves as the Erlichs eye-piece.

RESULTS

The reticulo-filamentous materials should be stained deep blue and the non-reticulated cells stained cliffuselly shades of pale-greenish blue.

COUNTING THE RETICULLOCYTES

An area of the film is chosen where the cells are undistorted and staining is good. The cells should not overlap.

Five hundred red cells are counted and the reticulocytes number is noted at the same time using a tally counter.

The reticulocytes count is calculated as follows:-

If N is the number of reticulocytes counted in 500 red cells, the reticulocyte count is

$$\text{Retics count} = \frac{N \times 100\%}{500}$$

NORMALS

Adults- 0.2-2%, Infants- 2-6%

QUESTIONS:

What are the causes of increased retics count?

What are the causes of decreased retics count?

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BLEEDING TIME TEST
IVY'S METHOD

Principle:
The duration of bleeding from standard puncture is measured. The bleeding depends upon the elasticity of the blood vessel wall and upon the number and functional integrity of platelets.

Reagents and materials:
- stop watch
- Sphygmomanometer cuff
- Filter paper
- Blood lancets
- Cleaning swabs.

Methods:

- A sphygmomanometer cuff is placed around the patients arm above the elbow and the pressure is maintained at 40mm of mercury.
- Swab the area and make three puncture with a lancet to the full depth of the needle on the flex or aspect of the forearm taking care to avoid visible veins start the stopwatch simultaneously with the first prick.
- Slot the drops of blood at 15-30 seconds intervals taking care not to touch the skin with the filter paper continue until the bleeding stops/ceases.
- Take the average the bleeding time of the three punctures.

DUKE'S METHOD

- Done on the earlobe mostly for infants,
- Place a towel on the shoulder to prevent blood accidentally dropping onto the patients clotting.
- Sterilize the patients the earlobe with spirit and puncture to a depth of about 2mm three places(3times)
- Start a stopwatch at the sane time as the puncture.
- Touch the formed drops of blood with the edge of a filter paper every 30 seconds, avoid touching the skin or the results maybe affected.
- Take the time when the bleeding stops for each puncture.
- If the bleeding time exceeds 15minutes apply pressure to the puncture to stop the bleeding and report as 15minutes plus.

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CLOTTING TIME (LEE AND WHITE METHOD)

Principle:

Whole native blood taken from a patient is incubated at 37oC water bath and timed till it clots while it's constantly checked.

Requirements:

- Water bath
- Test tubes
- Syringes and needles

Method:

1. Place 4 tubes in a water bath at 37oC.

2. Take venous blood with a clean dry syringe taking care that no juices enter the syringe. Start the stop watch immediately blood enters the syringe.
3. After detaching the needle deliver 1mm of blood into each four warmed tubes. Examine them at 30seconds interval and observe for clotting by gently tilting them through an angle greater than 90o without spilling the blood.
4. The clotting time is reported as the average of the time given by the four tubes.

Normal
5-11 minutes

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THROMBIN CLOTTING TIME

Principle:
The thrombin time reflects the reaction between thrombin and fibrinogen.

THROMBIN

FIBRINOGEN FIBRIN

It is prolonged when fibrinogen levels are very low in the presence of heparin and heparin-like substances, other inhibitors e.g. fibrinogen

degradation products (FDP'S) and when fibrinogen is qualitatively abnormal (dysfibrinogenaemia)

Method:

1. Pipette 01.ml of citrated plasma into a glass tube and incubate at 37oC for 1 minute.
2. Add 0.2ml of test thrombin reagent at 37oC and start a stop watch simultaneously.
3. Examine for clot formation.
4. Test in duplicate.

Results:

Prolongation of thrombin time suggests Hypofibrinogeniaemia, presence of heparin or heparin-like substances and decreased ability of fibrinogen to clot in fibrinocystics states.

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KAOLON CEPHALIN CLOTTING TIME (KCCT)/ ACTIVATED PARTIAL THROMBOPLASTIN TIME (APPT)

Principle:

Incubation of plasma with the optimal quantity of phospholipids and a surface activator leads to activation of the intrinsic coagulation system. The

addition of calcium chloride (CaCl2) triggers the coagulation process. This is a non-specific test of the intrinsic system. It is the most useful screening test for detecting deficiencies of factors VII,IX, XI and XII when taken together with a normal prothrommbin time.

Method:

- Place the tubes containing calcium chloride (M140) at 37oC for 5 minutes prior to use.
- Pipette 0.1ml control plasma into a glass test tube.
- Pipette 0.1ml of APPT reagent into the tube and mix.
- Incubate for 5minutes at 37oC
- Add 0.1ml M140 CaCl2 to the mixture and simultaneously start a stop watch.
- Examine for clot formation.
- Perform the tests in duplicate.
- The test plasma is treated likewise.

Results:

Are expressed in seconds
Reference range 30-40seconds,

Interpretation:

- A prolonged APPT with a normal PT indicate possible deficiency of factor VII, IX, XI, XII high molecular weight kininogen, Prekarliklein or the presence of an inhibitor.
- APPT will also be prolonged in any deficiency involving common pathways (deficiency of factor V, X, II and to a lesser extent fibrinogen)
- The presence of some therapeutic inhibitors of coagulation such as heparin will also prolong APPT.

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HAEMAGLOBIN ESTIMATION

1. **ACID HAEMATIN (SAHLI) METHOD,**

Requirements

- Sahli tube,
- Standard tube,
- Stirring rod,
- 0.02ml pipette,
- Sucking tube,
- Pasteur pipette,
- 0.1N hydrochloric acid.

PRINCIPLE

Haemoglobin is converted to acid haematin by action of 0.1N HCL which is thereafter matched with the standard.

TECHNIQUE

- Fill the sahli tube up to the 20^{th} mark with 0.1N HCL,
- Add 0.02mls of blood using the Hb pipette,
- By use of a Pasteur pipette add either 0.1N HCL or distilled water drop wise, mixing between each dilution with the stirring rod, until the colour matches that one for standard.
- Read the amount of solution in the sahli tube directly and report either in grams or %,
  E.g. Given N% converts to grams.

  $\frac{N}{100} \times 14.6$ g/l

  Gms% = gms/100mls

  N/B 14.6 gms/100mls is the standard concentration.

Questions:

1. List 5 advantages of method,
2. List disadvantages of the method.

2. ALKALINE HAEMATIN METHOD,

Requirements:

-Clorimeter with green filter,
-Haemoglobin standard (=16.0%)(Gibson and Harrison),

-0.05 pipette,
-0.1M NaOH,
-Test tubes.

PRINCIPLE

Haemoglobin is converted to alkaline haematin by the action of Sodium hydroxide. The standard is commercially obtained and is 16gm%.

$$\frac{\text{OD Test}}{\text{OD Standard}} \times \text{Conc. of standard}$$

METHOD,

- Add 0.05ml of blood to 4.95mls of N/10 NaOH in a test tube,
- Mix well and boil for 4min at the same time boil 5mls of standard solution,
- Cool both tubes quickly in cold water and match the test against the standard using a colorimeter with a green filter,
- Calculate the Hb contents in grams per 100mls of blood.

Colorimeter test reading = 21
Colorimeter standard reading = 28
Standard = 16gm/100mlsHb,

Therefore test Hb = $\frac{21}{28} \times 16 = 12\text{gm}/100\text{mls}$

As 14.6gms/100mls = 100% then,
Hb = $\frac{12}{14.6} \times 100 = 82\%$

QUESTIONS,

1. List 2 advantages of the test,
2. List 4 advantages of this test.

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HB ELECTROPHORESIS

Requirements,

- Cellulose acetate paper (medium), Stains e.g. methylene green,
- Specimen mostly haemolysate, 1-2% Sulphosalicylic acid,
- Buffer, Stds. in the form of haemolysate and they include HbAA, HbAs, HbSf,
- Source of power, Electrophoretic tank.

PRINCIPLE

It is based on the fact that haemoglobin being proteins, when subjected to electric current in a buffer it acquire charges and begin to migrate and the speed depends on the number of charges in each haemoglobin e.g.HbA acquires 5 charges, HbF acquires 3 charges, HbS acquires 2 charges.
HbA is the fastest and HbS is the slowest.

PROCEDURE

1. Prepare a Haemoglobin of the test sample by,
    - 1ml blood collected in EDTA,
    - Wash 3 times in normal saline to remove other proteins,
    - Add equal amount of water to packed cells to lyse the cells,
    - Shake the tube hard,
    - Add 1/3 of volume of Carbon tetrachloride to remove the cell stroma,
    - Shake again,
    - Centrifuge lightly for 3min,
    - Use Pasteur pipette to separate reddish haemolysate from the stroma,
    - Use haemolysate for the test.
    -
2. Prepare the cellulose acetate paper,
    - Draw a line with a pencil 1cm from the edge,
    - Label according to specimens you have including all the standards,
    - Soak the CAPE in buffer solution and plot it to remove excess buffer solution,
    - Innoculate all your test and standards,
    - Fix paper on the electrical tank and switch on the current for about 20min,
    - Remove paper and stain with methyl green,
    - Decolourise with 2-3% Glacial acetic acid,

- Hang it on vertical surface to dry,

+ -

AA

1 A^1

AS

S^1 A^1

2

SS

S^1

3

SF

S^1 F^1

X

Hb F ESTIMATION

REQUIREMENTS

- 0.1 N NaOH,
- 1/2 saturated acidified ammonium sulphate solution,
- 0.04% ammonium solution,
- Clorimeter with a cuvette,
- Filter of 540nm,
- 10ml pipette,
- 0.2ml pipette,
- 5ml pipette,
- Filter paper,
- Test tubes.

PRINCIPLE

It is based on the fact that HbF unlike other haemoglobin resist alkaline denaturation.

PROCEDURE

- Pipette 3.2ml of NaoH solution in a test tube,
- Add 0.2ml of blood and mix,
- Start a stop watch immediately,
- After exactly 1min add 6.8mls of the half saturated acidified ammonium sulphate solution (to stop the reaction),
- Filter the solution and take the OD of filtrate,
- Use distilled water as a blank.

STANDARD SOLUTION

- Put 0.2mlsof blood in a test tube,
- Add 0.04mls ammonium solution,
- Take the OD directly,
- OD of standard.

RESULTS

Get the % of Hb F in a specimen by,

$$\frac{\text{OD of test}}{\text{OD of Standard}} \times 100$$

That will be % of HbF.

Normals,

Adults 1-2%
Infants 95-98%

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HAM'S TEST (ACID – SERUM TEST)

PRINCIPLE

When serum is acidified it enhances complement development binding to red cells. PNH cells are more susceptible to haemolysis under such conditions of optimum Ph of 6.5 – 7.0.

METHOD

- Put 0.5mls fresh normal serum (ABO compatible with patients cells) into 6 test tubes,
- Incubate 2 tubes at 56^{o}C for 30min (to inactivate complement) and the others at RT,
- Add 0.05ml of HCL (0.2N) to all the tubes,
- Add 0.05mls 50% washed patients cells into each acidified tube and incubate at 37^{o}C for an hour,
- Spin and check for haemolysis using a colorimeter at 540nm.

RESULTS

PNH case-----Cells in acidified tubes will definitely lyse or even more visible in unacidified inactivated tube.
NEGATIVE----No lysis with the acidified inactivated tube.
CONTROL CELLS----Normal control cells shouldn't lyse in any of the three tubes.

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SUCROSE HAEMOLYSIS TEST

REQUIREMENTS
Sugar water—Commercial granulated sugar -10ml.
Distilled water----------100ml,

PRINCIPAL
Red cells of PNH patients lyse when subjected to low ionic strength solution in compliment present.

METHOD
- Add 0.05ml normal serum to 0.85ml fresh sugar water and mix,
- Add 0.1ml 50% cells suspension,
- Mix and incubate at RT for 30min,
- Centrifuge and examine supernatant for haemolysis,
- Read spectrophotometrically at 540nm and calculate percentage.

N/B,

1. Zero solution -- 0.05ml normal serum + 0.85ml sucrose solution.
2. 100% Haemolysate – 0.05ml serum + 0.85ml sucrose solution + 0.1ml cell suspension.
3. Patients Haemolysis – 0.05ml supernatant from incubated reaction mixture + 5ml Drabkins solution.

CALCULATION

$$\frac{\text{Patients Haemolysis OD}}{\text{100\% Haemolysis OD}} \times 100 = \% \text{ Sucrose Haemolysis.}$$

INTERPRETATION,
1. 5% Haemolysis or less = NORMAL,
2. 5-10% Haemolysis = Borderline,
3. >10% Haemolysis = PNH.

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LE CELLS DEMONSTRATION

REQUIREMENTS,

- Glass beads,
- Test tube,
- Blood.

METHOD,

- Transfer 1ml of blood into a 75 x 12mm glass tube,
- Add 4 glass beads and seal the tube tightly,
- Rotate the tube at 33rpm for 30min,
- Incubate the blood at 37^{o}C for 10-15min,
- Transfer the contents of the tube into a Wintrobe tube and centrifuge for 10min at 1000-1500rpm,
- Make buffy coat films from the wintrobe tube and allow the film to air dry,
- Stain by leishman stain.

RESULTS,

The LE cells appeases a neutrophil in the cytoplasm of which is a large spherical body (the LE body) which stains shades of pale purple and it appears as an opaque homogenous mass.

MLS 2310: BLOOD TRANSFUSION

BLOOD GROUPING

Principle:
Make use of standard, known antisera (containing agglutins or antibodies) to detect unknown antigens (agglutinogens) on the surface of the red cell membrane thus defining the red cell blood group.
Antisera usually contain antibodies.

Requirements:

1. Antisera – A, B, AB, D
2. Standard cells -5% suspensions in saline of A cells, B cells, O cells, AB cells.
3. Test cells suspension.
4. Test tubes
5. Tiles

TUBE METHOD

- Forward and reverse grouping.

1. Label tubes Anti-A, Anti-B, Anti-D, A-cells, B-cells, O-cells and AB-cells.
2. Add one volume of antiseras in the respective tubes.
3. Add one volume of 4% cells suspension of each to respective tubes.
4. Add one volume of 4% test cells suspension in tubes with antiseras.
5. Add one volume of test serum in each of the tubes with 4% known cells suspension.
6. Mix the contents in each tube and spin at 1000RPM for 1minute.
7. Examine the tubes both macroscopically and microscopically.

Results:

Anti-A	Anti-B	Anti-D	A-cells	B-cells	O-cells	Blood group
+	-	+	-	+	-	A+Ve
-	+	+	+	-	-	B+Ve
+	+	+	-	-	-	AB+Ve
-	-	+	+	+	-	O+

TILE METHOD

- On a clean tile add one drop of respective antiseras and known cell suspension.
- Add a droop of test cells suspension and/or test serum.
- Mix and observe for presence or absence of agglutinations.

- Results are as above.

MLS 2310: BLOOD TRANSFUSION

DU TEST

Principle:
DU antigen is weak and cannot react with anti-D directly, so DU sensitized antigens are sensitized and AHG acts as a bridge to make them visible.

Requirements:
- Test tubes
- Anti-D
- Test cells
- AHG (Antihuman Globulin)

Method:
- Label test tubes as Test, Positive control and Negative control.
- Add 1 drop of known standard anti-D to each tube.
- Add 1 drop of 5% suspension of patient/donor cells to the tube labeled test, one drop of 5% suspension of group O, +ve cells to positive control and one drop of 5% suspension of O-ve cells to the negative control.
- Mix and incubate for 30-60minutes at 37oC.
- Spin and check for agglutination.
- Wash three times with normal saline.
- Drain the supernatant and add three drops of AHG.
- Mix and spin at1000RPM for 2 minutes agglutination both macro and microscopically.

Results:
If agglutination is present report as DU test positive,
If agglutination is absent report as DU test negative

MLS 2310: BLOOD TRANSFUSION

ANTIHUMAN GLOBULIN TEST (COOMBS TEST)

There are two types of AHG tests depending on whether the red cell sensitization takes place invivo (inside the body) or invitro (outside the body)
-Invitro the test employed is called Indirect AHG
- Invivo the test employed is called Direct AHG.

DIRECT COOMBS TEST (DCT)

Principle:
If there is in vivo sensitization of the red cells as in case of Haemolytic Disease of the New born, incompatible transfusion reaction. Auto-Immune Haemolytic Anaemia etc the sensitized cells will be agglutinated by globulin antibodies by the use of AHG serum.

Requirements:

- Test tubes
- Anti-Human globulin
- Patient cells.

Method:

- Label a test tube DC (direct coombs)
- Add about 2 drops of patient cells and wash at least 3 times in large volumes of normal saline. After the last wash discard the saline completely.
- Add one drop of AHG, centrifuge at 1000RPM for 1minute and examine both macro and microscopically for presence of agglutination.

Interpretation:
If agglutination is present report as DCT +ve,

If agglutination is negative report as DCT-ve,
Agglutination indicates that the cells have been sensitized in the body.

MLS 2310: BLOOD TRANSFUSION

MAJOR CROSS MATCH

Requirements:

- Test tubes
- Donors cells
- Patient serum
- AHG
- Albumin

Principle

To detect antibodies in the patient serum which correspond with donor cells.

Procedure

Group the patient and select a suitable donor. The donor should be of the same blood group to the patient.

- Wash the donor's cells 3 times after which you discard the last wash completely and make 4%cell suspension in normal saline.
- Label 4tubes SRT, S37, A37 and C37.
- Add 2 drops of the patient's serum to each of the tubes.
- Add equal volume of 4% cell suspension of donor cells in each tube and mix well.
- To the tube labeled A37 add albumin on the side of the tube so that it goes down to displace the cells and settle down.(don't mix)
- Do an immediate spin on the SRT tube so as to check for AOB incompatibility. If no incompatibility, proceed with the test and if there is incompatibility stop the test immediately.
- Incubate the tubes at respective temperatures for 45minutes.
- Spin lightly the SRT, A37 and S37 tubes and observe for the presence of agglutination.

- Wash C37 tube 3 times in large volumes of normal saline, discard the supernatant in the last wash completely then add 1 volume of Antihuman Globulins and mix.
- Spin lightly for 1minute and observe for presence of agglutination both macro and microscopically.

Report
If agglutination at any phase then the cross match is incompatible.
If no agglutination at any phase then the cross match is compatible.

MLS 2310: BLOOD TRANSFUSION

MINOR CROSS MATCH
The procedure is the same as that of major cross match except that the specimens used are donor serum and patients cells.

Procedure
Label 2 tubes A37 and SRT.
- Add 2 drops of 4% patient's cells to each tube.
- Add 2 drops of donor serum in to each tube.
- To the tube labeled A37 add bovine albumin and incubate at 37oC for 45minutes.
- Do an immediate spin to tube SRT then incubate at room temperature for 45 minutes.
- Centrifuge both tubes and read macro and microscopically.
- For the tube A37 wash 3times in large volumes of normal saline and add 1drop of AHG.
- Incubate for further 15-30minutes at 37oC.
- Spin at 1000rpm and observe for the presence or absence of agglutination.

Questions:
(1) List 3 causes of incompatible cross match.
(2) What are the causes of error in cross matching?

MLS 2310: BLOOD TRANSFUSION

ANTIBODY SCREENING TEST

Purpose
The purpose is to find out the causative antibody in the serum. Problem would have arise through a cross match incompatibility or any haemolytic phenomena before antibody screening is done. Thus it also tells us at what temperature at what phase of the medium it reacts best. Usually we have six phase antibody screening namely;

-4^{o}C phase – S 4^{o}C - Albumin- A37
-Coombs - C37Enzymes- E37
-Room temperature- SRT - Saline at 37oC- S37

Requirements:
- Test tubes.
- 'O' +VE cells.
- Albumin

Principle
'O' positive cells react with the offending antibodies to show agglutination which is viewed macro and microscopically,

Procedure

- Label 6 tubes as 4°C, SRT, S37, A37, C37 and Enzymes.
- Add 2 drops of test serum to all the tubes.
- Add equal volumes f 4% 'O' positive cells to all the tubes and mix.
- Add an equal volume of albumin to the A37 tube and Enzyme to the tube labeled enzyme.
- Incubate all the tubes in respective temperatures for 1-2hours.
- Wash the AHG (C37) tube 3times in large volumes of normal saline and discard the supernatant of the last wash completely.
- Add 1volume of AHG and mix to resuspend the cells.
- Spin all the 4tubes at a low speed and observe for agglutination.
- Note the phase and the medium at which agglutination occurs if any.
-

Some antibodies are specific and only react at one given phase.
4oC- Expect cold antibodies I and P
RT- ABO antibodies particularly those of IgM.
S37oC- Strictly antibodies of the rhesus group.
A 37oC- Rhesus antibodies and some of the irregular antibodies.
C 37oC- Irregular antibodies,

Questions

- What is the difference between antibody screening and cross matching.

MLS 2310: BLOOD TRANSFUSION

TITRATION OF ISO-AGGLUTININS

Purpose:

This is to detect the strength of antibodies which is used to determine the severity of some diseases e.g. haemolytic disease of the newborn.

Procedure:

- Label 10test tubes as neat, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256 and 1:512.
- To each tube add 2 volumes of saline except in the neat tube.
- Add 2 volumes of patient serum in tubes 1 and 2.
- Mix the contents in tube 2 and transfer 2 volumes to tube 3.
- Mix the contents in tube 3 and transfer 2 volumes to tube 4.

Continue with this process of serial dilution until you discard 2 volumes from tube 10.

- Add 2 volumes of 4% known cell suspension according the antibody being titrated to all tubes.
- Incubate at room temperature or respective temperature for 1-2 hrs depending on the antibodies being titrated.

- Centrifuge all tubes at low speed and observe for the last tube showing agglutination.

Interpretation:

The titre of the antibody is given by the last tube which shows agglutination.

MLS 2310: BLOOD TRANSFUSION

ANTIBODY IDENTIFICATION

Requirements

-Panel of known cells,
-Test tubes.

Purpose

To find out the reacting antibody and classify them,

Method

1. Use a panel of known cells (commercial cells) i.e. they have known antigen.
2. These cells are added to labeled tubes.
3. Equal volume of the test serum is added to each tube.
4. Incubate at the appropriate temperature (i.e. the temperature at which it reacted when doing antibody screening).
6. Examine for agglutination, incase it reacts,

1 2 3 4 5 6

+ - - + - +

RoRo	R2R2	R2r”	r’r”	ry ry	rr
+	-	-	+	-	+
Dce/Dce	DcE/DcE	DCE/dcE	dCe/dcE	dCe/dcE	dcE/Dce

The antibody causing this problem is anti –e

The antibody causing these problems is anti-C

R=D	r=d
R_1=C	r^i=c
R_2=E	r^ii=e
R_2=DCE	r^y=dce

MLS 2310: BLOOD TRANSFUSION

ANTIBODY TITRATION

Requirements:

-Test tubes,

Purpose:

To find out the strength of the antibody, Used to determine the seriousness of some diseases or abnormalities e.g. haemolytic disease of the newborn.

The common media in which titration is done are,

- Saline,
- Albumin,
- AHG
- Enzymes

METHOD USED IN SALINE MEDIUM,

1. 10 Tubes are labeled,
2. Add one volume of saline from the second tube to the test tube,
3. Add serum to the first and the second tube one volume each,

4. Mix the content in tube two and transfer one volume to tube three, continue with the serial dilution to the last tube, then discard one volume.
5. Add one volume of the test cells to all the tubes, that is 4% cell suspension (use known cells and known serum e.g. A cells and anti-A).
6. Mix, then incubate at the required temperature.
7. Observe for agglutination macroscopically.

Interpretation:
The last tube which shows agglutination is the one taken as an indicator of that serum.

MLS 2310: BLOOD TRANSFUSION

INDIRECT COOMBS (TEST AHG)

This is a test used to find or detect those cells that are sensitized in vitro (outside the body)
It is used in cross-match antibody screening tests etc

Principle
If there is incomplete antibody in the patient's serum and you add the corresponding red cells, these cells become sensitized by the incomplete antibody. When you add the AHG the sensitized cells are joined together thus the reaction becomes visible.

Method

1) Prepare a 4% pooled O-positive cells suspension in large volumes of saline.
2) To one volume of the 4% O-positive cells, add one volume of the patient's serum or vise varsa.

3) Mix and incubate for 30-60 minutes at 37oC.

4) Wash the contents of the tube at least 3 times in large volumes of saline. After the last wash discard the last wash completely.

5) Add one volume of AHG and mix the contents.

6) Spin at 1000rpm for 1minute after which you examine both macro and microscopically for agglutination.

If agglutination is present report as positive indirect coombs test (ICT +ve)

If agglutination is absent report as negative indirect coombs test (ICT – ve)

Interpretation:

If there is agglutination it means that there is an incomplete antibody in the patient's serum – identify the antibody by use of panel cells.

PARASITOLOGY

MLS 2201: GENERAL PARASITOLOGY

EXAMINATION OF STOOL SAMPLES USING DIRECT SALINE PREPARATION METHOD

INTRODUCTION

For clinical purposes, a fresh faecal specimen is required. It should be uncontaminated with urine and collected into a suitable size clean, leak proof container. The acceptable size of stool should be pigeon pea size.
Stool should be processed immediately on arrival at the laboratory. The following is recorded on the container;

- Time the patient passed the stool.
- Patient number.
- Date of collection.

The direct examination of faeces is essential to detect motile parasites and is usually adequate significant helminthes infection

Macroscopic examination

Report on the following:

- Colour of the specimen – brown, black, green e.t.c
- Consistency- formed, semi formed, unformed, and watery.
- Presence of blood, mucous or pus
- Whether the specimen contains worms or worm segment

Microscopic examination

This reveals trophozoites of pathogenic Entamoeba histolytica, Giardia lamblia, Balantidium coli, and cysts of pathogenic protozoa.

Intestinal amoeba belongs to the following genera:-

- Entamoeba – e.g. Entamoeba histolytica
- Iodalimax – e.g. I. buschili
- Endolimax - e.g. E. nana
- Dietamoeba – e.g. D. fragilitis

Materials

Microscopes
Glass slides
Cover slips,
Applicator sticks
Physiological saline
Lugol's iodine

Method

1. Place a drop of physiological saline on one slide and a drop of iodine on another slide
2. Using an applicator stick, mix a small amount of specimen (about 2mg) with the saline and a similar amount with the iodine
3. Make smooth thin preparations and cover each preparation with a cover glass
4. Examine systematically the entire saline preparation for larvae, ciliates helminthes eggs, cysts and oocysts. Use the10x objective with the condenser iris closed to give a good contrast
5. Use the 40x objective to assist in the detection and identification of eggs, cysts and oocysts. Always examine several microscopic fields with this objective before reporting 'NO' parasites found

6. Use the iodine preparation to assist in the identification of cysts.

Note: -
Iodine kills living organisms in stool and must be done after direct saline examination has been completed.

Exercise
Draw and label any ova or cyst seen

MLS 2201: GENERAL PARASITOLOGY
FORMAL ETHER SEDIMENTATION TECHNIQUE

Sedimentation techniques have been used as one of the best method in diagnosis of worm infections. The parasites are sedimented by gravity or centrifugal force and therefore a wide range of parasites can be concentrated within minimum damage to the morphology.
Solutions of high specific grafity i.e.solution that is denser than the parasites being concentrated.

Principle:
Faeces are emulsified in formal water, the suspension is strained to remove large faecal particles, ether is added and the mixed suspension is centrifuged. Cysts, oocyst, eggs and larva are fixed and sedimented and the faecal debris is separated in a layer between the ether and formal water. Faecal fat is dissolved in the ether.

Requirements:

-Formal water 10% v/v
-Strainer (with small holes 400-450)
-Diethyl ether
-Rods

-Beaker
-Centrifuge tube
-Slides cover slips
-Screw cap bottle or tube
-Pipettes
-Gauze
-Iodine

Method

- Using a rod, emulsify an estimated 1g (pea size) of faeces in about 4mls 10% formal water contained in a screw cap bottle or tube.
- Add further 3-4 mls of 10 % formal water, cap the bottle, and mix well by shaking.
- Sieve the emulsified faeces, collecting the sieved suspension in a beaker.
- Transfer the suspension to a centrifuge tube.
- Add 3- 4 mls diethyl ether.
- Stopper the tube and mix for 1 minute.
- With tissue wrapped around the top of the tube, loosen the stopper.
- Centrifuge immediately at 30000 rpm for 1 minute.
- Using a stick, loosen the layer of faecal debris from the side of the tube and invert the tube to discard the ether, faecal debris and formal water. The sediment will remain
- Return the tube to its upright position and allow the fluid from the side of the tube to drain to the bottom. Tap the bottom of the tube to resuspend and mix the sediment.
- Transfer the sediment to a slide and cover with a coverslip.
- Examine the preparation microscopically using x10 objective with the condenser iris closed sufficiently to give good contrast.
- Using x40 objective to examine small cysts and eggs.
- To assist in the identification of cysts, run small drop of iodine under the cover glass.

NB:/The number of each species of egg in the entire preparation can be counted. This gives the approximate number per gram of faeces

MLS 2201: GENERAL PARASITOLOGY

ZINC SULPHATE FLOATATION TECHNIQUE

A zinc sulphate solution is used which has a specific gravity of 1.180- 1.200. Feaces are emulsified in the solution and the suspension is left for the eggs and cysts to float to the surface. They are then collected on a cover glass. This technique is recommended for concentrating the cysts of G. lamblia, E. histolytica, and the eggs of T. trichuria, opisthorchis and clonorchis

Requirements

Zinc sulphate solution
Test tube

Strainer

Method

- Fill the tube about one quarter full with the zinc sulphate solution. Add an estimated 1 gram of faeces (2ml if in liquid form). Emulsify the specimen in the solution.
- Fill the tube with the zinc sulphate solution and mix well. Strain the faecal suspension to remove the large debris.
- Return the suspension into the tube. Stand the tube in a completely vertical position in a rack.
- Using a plastic bulb pipette add further solution to ensure the tube is filled to the brim.
- Carefully place a completely clean cover glass on top of the tube, avoid trapping air bubble
- Leave undisturbed for 30-40 minutes to time for the ova and cysts to float
- Carefully lift the cover glass from the tube by a straight pull upwards. Place the cover glass facing downwards on a slide.
- Examine microscopically the entire preparation using the 10X objective with the condenser iris closed sufficiently to give good contrast. Use the 40X objective, and run a drop of iodine under the cover glass to identify the cysts.
- Identify any parasites present.

MLS 2201: GENERAL PARASITOLOGY
DEMONSTRATION OF PARASITES IN A BIOLOGICAL SPECIMEN

NEMATODES

Nematodes are cylindrical worms with a body cavity and a cuticle which may be smooth, spined, and ridged. Most of the medically important intestinal nematodes are geohelminthes; a person becomes infected by swallowing infective eggs e.g. A. lumbricoides, T. trichiura, E. vermicularies or by infective larvae penetrating the skin e.g. hookworms.

A.lumbricoides

- Freshly expelled Ascaris worms are pinkish in colour with an appearance similar to earth worms.
- They measure 12-15 cm in length and taper at both ends. The tail of the male is curved and has small rod like projections. (Spicules).
- There is a small mouth surrounded by three lips.

CESTODES

Cestodes are segmented and tape like, it consists of a head (scolex)and many proglottids (segments), the is no body carvity.

The scolex has suckers and in some species also hooks that attach the tape worm to its host, there is no mouth or digestive system, it obtains its nutrients through its body surface. They include; Taenia species, E. granulosas, D.latum, H. nana.

T. saginata segments

- Appears white and opaque and measures about 20mm long by 6mm wide when freshly passed. It's longer than T. solium segments.
- Uterus has a central stem which has more than 13 main side branches on each side (T. solium has fewer than 13) the main side branches are sub divided into smaller branches.
- The scolex is very small measuring only 2mm across, the scolex has four suckers but no hooks

T. solium segments

- Appears grey blue and translucent and measures about 13mm long by 8mm wide when freshly voided, its shorter than a gravid segment of T. saginata.
- Uterus has a central stem which has up to 13 main side branches on each side, main side branches sub divided into in to smaller branches

HELMINTHES

Liver flukes

- Flukes are un segmented mostly flat leaf like worms, without a body carvity.
- They have oral and ventral suckers.
- The digestive system consists of a mouth and an esophagus which divides to form two intestinal caeca
- There is no anus but an excretory system composed of excretory cells, collecting tubules, and an excretory pore.
- There is a simple nervous system

MLS 2201: GENERAL PARASITOLOGY

GIEMSA STAINING TECHNIQUE

Giemsa stain is an alcohol based rowmanowsky stain that requires dilution in pH7.1-7.2 buffered water before use. It gives the best staining of malaria

parasites in thin films. It also stains thick films well provided they are completely dry, the concentration is low and the staining time is sufficiently long. Less satisfactory results are obtained when the concentration of the giemsa stain is increased to reduce the staining time.
Staining is a method of imparting colour on cell organelles or their background to enable easy identification of different characteristics of cells and micro-organisms.

Materials required

- Prepare thick and thin films
- Giemsa stock solution
- Leishman stain
- Phosphate buffer
- Measuring cylinders
- Oil immersion
- Lancets
- Microscope

GIEMSA STAINING TECHNIQUE (THICK FILMS)

Preparation of stock solution
-Giemsa powder 3.8gm
-Absolute methanol 250ml
-Pure glycerol 250ml
Dissolve the powder in the alcohol in a dry conical flask. Stir using a glass rod until the powder has completely dissolved.
Add the glycerol and store in a labeled reagent bottle.

WORKING SOLUTION
Use 5% or 10% of giemsa stock solution diluted in phosphate buffer pH 7.2.

Preparation of phosphate buffer
Disodium hydrogen phosphate powder (Na_2HPO_4) 0.7gm
Potassium dihydrogen phosphate powder (KH_2PO_4) 0.3gm
Distilled water 1000ml
Weigh the powder or use buffer tablets pH 7.2 and dissolve in distilled water.

To prepare 5% giemsa solution
Giemsa stock solution 5ml
Phosphate buffer pH 7.2 95ml
Mix the 2 solutions in a measuring cylinder.

10% Giemsa solution
Giemsa stock solution 10ml

Phosphate buffer Ph 7.2 90ml
Mix the 2 solutions in a measuring cylinder.

METHOD OF STAINING

1. Place the slides on the staining rack.
2. Flood the slides with the diluted giemsa solution. Leave the stain as follows;
   5% giemsa for 20 minutes.
   10 % giemsa for 10 minutes.
3. Wash off excess stain with a thin stream of phosphate buffer. Distilled/ clean water may be used and allow the slides to drain dry and examine them under a microscope.

MLS 2201: GENERAL PARASITOLOGY

PRESERVATION OF PARASITES AND PREPARATION OF PERMANENT MOUNTS

INTRODUCTION

The preservation of parasites and preparation of permanent mounts of parasites in specimens are required;

- For the control of microscopical parasitological tests and for training purposes
- When specimens are sent to peripheral laboratories as part of an external parasitological quality assessment programme
- When specimens need to be sent to a reference laboratory for parasite identification
- When specimens are collected in the field during epidemiological surveys

Storage and preservation destroys blood cells, macrophages, motile bacteria, trophozoites of protozoa and preserves well the morphology of feacal eggs and cysts

Requirements

Formaldehyde	40%
Sodium chloride	
Applicator sticks	
Measuring cylinders	100 mls
Stool sample	
Universal bottles	
Weighing balance	
Gummed labels	
Reagent bottles	
Distilled water	

Preparation of 10% formal saline

Formaldehyde 40%	10mls
Sodium chloride	0.85gms
Tap water	90.0mls

Weigh 0.85gms of sodium chloride
Dissolve sodium chloride in some amount of tap water. Add 10mls of 40% formaldehyde
Mix well and add the remaining water
Mix well, label and store at room temperature.

Method

- Place a small of the stool sample in a clean universal bottle
- Add an equal volume of 10% formal saline to the stool sample
- Mix well with an applicator stick and cap tightly
- Label the bottle clearly with the patient name, age and date of collection
- Store the preserved stool samples at room temperature. May be stored for up to three months

Permanent mounts

Malaria parasites, trypanosomes, microfilariae, leishmania amastigotes and other parasites in stained smears are best preserved by using a mountant such as DPX. Such preparations can be kept for several years with good preservations of parasite morphology and little fading of the stain, loss of transparency, or shrinkage of the mountant

Procedure

- Place a drop of mountant on the dry oil free film or smear
- Cover with a cover glass and allow to air dry
- When dry peel off the excess mountant

MLS 2201: GENERAL PARASITOLOGY

FIELD STAINING TECHNIQUE

This water based rowmanowsky stain is composed of two solutions, Field stain A and Field stain B. It's buffered to the correct PH and neither solution requires dilution when staining thick films. When staining thin films, field stain B requires dilution. Field stains are more stable, they stain fresh blood well particularly thick films. The rapid technique is ideally suited for staining blood films from waiting outpatients and when reports are required immediately

Materials

- Clean glass slides
- Blood sample
- Immersion oil
- Field stain A & B
- Microscope
- Absolute methanol

METHOD:

(A). Thick Films

Procedure

Prepare thick films as required

Arrange four coupling jars on the bench, label

Stain by dipping the slides in the jars as follows:

- Field stain A-4 seconds
- Clean water- 5 seconds
- Field stain B- 4 seconds
- Clean water- 5 seconds

Allow the slides to air dry and observe using oil immersion

THIN BLOOD FILMS

Reverse staining technique is normally used to stain thin films. The films are first fixed with absolute alcohol then staining with field stain B followed by field stain A.

The technique preserves all blood cells and parasites to be examined

Procedure
Prepare thin films as required
Fill a jar with absolute methanol
Fix the films by dipping in absolute methanol for 1-2 seconds
Stain by dipping the slides in the jar as follows

- Field stain B – 4 seconds
- Clean water - 5 seconds
- Field stain A – 4 seconds
- Clean water - 5 seconds

Allow the slide to drain dry.

Examine using oil emersion objective of the microscope, for trophozoite (ring forms not gametocytes)

MLS 2302: CLINICAL PROTOZOOLOGY
LABORATORY TECHNIQUES AND METHODS

EXAMINATION OF INTESTINAL PROTOZOANS USING DIRECT SALINE PREPARATION METHOD.

INTRODUCTION

Stool is collected in sterile containers (polypots) and is immediately processed on arrival at the laboratory. The following is recorded on the container;

- Time the patient passed the stool.
- Patient number.
- Date of collection.

The acceptable size of stool should be pigeon pea size. Stool is examined visually immediately for consistency as formed, liquid etc. Colour as white, yellow, and brown, black. Microscopic examination of stool reveals trophozoites of pathogenic entamoeba histolytica, giardia lamblia, balantidium coli, and cysts of pathogenic protozoa.

Intestinal amoeba belongs to the following genera:-

- Entamoeba – e.g. Entamoeba histolytica
- Iodalimax – e.g. I. buschili
- Endolimax - e.g. E. nana
- Dietamoeba – e.g. D. fragilitis

Materials

Microscopes, glass slides, cover slips, applicator sticks, physiological saline, and lugol's iodine.

Method

1. A small amount of stool is mixed with a drop of physiological saline on a glass slide using an applicator stick. Examine under a microscope.
2. Iodine preparation- for cysts of protozoa. Note: - iodine kills living organisms in stool and must be done after direct saline examination has been completed.

- Place a drop of lugol's iodine on a clean slide. Take a small sample of stool with applicator stick and mix with iodine on slide. Put a cover slip avoiding air bubbles and observe under x10 objective for cysts.
- Examine for more details with x40 objective.

Draw and label.

MLS 2302: CLINICAL PROTOZOOLOGY

LEISHMANIA

INTRODUCTION

Leishmania donovani is a protozoan that causes visceral leishmaniasis. Visceral leishmaniasis is diagnosed by demonstrating leishmania in field stained preparations of splenic aspirate, lymph gland fluid and bone marrow aspirate (in specialized centres). A splenic aspirate is the preferred sample. Leishmania donovani is transmitted by sand flies *(phlebotomus* species). The infective form of parasite promastigotes are introduced into man by infected sand flies during blood meal.promastigotes develop into amastigotes which multiply in large numbers and are carried to the internal organs mainly liver, spleen, and bone marrow where they are taken up by macrophages. Sand flies become infected when they feed on blood containing amastigotes. In sand fly gut, amastigotes continue to multiply and develop into promastigotes. Samples of splenic tissue should only be collected by a clinician. Films should be prepared and stained immediately after collection of samples.

Materials
Stained slides, microscope, and oil immersion

Method
Place the slide on the microscope stage, swing the x10 objective into position and focus. Swing the x40 objective into position. Examine the whole film systematically for parasites. Examine in detail using x100 oil immersion objective. Record the findings and draw large well labeled diagrams of your observation.

MLS 2302: CLINICAL PROTOZOOLOGY

HAEMOPARASITES

Introduction

Haemoparasites are blood parasites and they include plasmodium species, trypanosomes, and microfilariae. Trypanosomes are haemoflagellates. All members of genus trypanosomes exists at some time of their life cycle as trypomastigotes with an elongated spindle shaped body, a central nucleus, a posterior flagellum and a long undulating membrane. Human infection is acquired by the bite of the vector tse-tse fly. The infective form of the parasite is the metacyclic trypomastigote. In blood, 3 forms of trypanosomes are found; the long slender trypomastigote, a short broad form with a flagellum attenuated or absent and an intermediate form. Trypomastigotes are about 15-30microns long and 1.5-3.5microns broad. In fresh film, they may be seen as colourless spindle shaped bodies that move rapidly spinning red cells around. Cytoplasm appears pale blue and nucleus red with smears stained with the giemsa or other romanowsky stains.

Materials

Microscope and prepared permanent glass slides.

Method

Place the glass slide on the stage of the microscope & examine first using the low power and then higher power objectives for more details.
Draw and label the parasites seen.

MLS 2302: CLINICAL PROTOZOOLOGY

INTESTINAL PARASITES

Introduction

Intestinal parasites include:
-Platyhelminthes –flatworms/trematodes.
-Nematohelminthes- roundworms/nematodes.
-Acanthocephalus- cestodes.
The first and second group has free living members but the third are fully parasitic.

1. Nematodes are cylindrical and unsegmented. They is dioecious (different sexes). Adults produce eggs which hatch or releases larvae.

2. Cestodes are usually segmented and each segment contains a set of both male and female reproductive system. The body is divided into three parts; head, scolex and proglottids.

3. Trematodes are flattened dorsal ventrally and all use snails as host vector. They are all hermaphrodites except the schistosomes.

Materials
Microscope, prepared permanent slides and preserved specimens.

Method
Place the slide on the microscope slide. Examine the preparation.
Examine macroscopically the provided preserved specimen.
Draw and name the parasites seen.
List the distinguishing characteristics.

MLS 2302: CLINICAL PROTOZOOLOGY

FORMAL ETHER SEDIMENTATION TECHNIQUE

Sedimentation techniques have been used as one of the best method in diagnosis of worm infections. The parasites are sedimented by gravity or centrifugal force and therefore a wide range of parasites can be concentrated within minimum damage to the morphology.

Solutions of high specific grafity i.e.solutions that are denser than the parasites being concentrated.

Principle:

Faeces are emulsified in formal water, the suspension is strained to remove large faecal particles, ether is added and the mixed suspension is centrifuged. Cysts, oocyst, eggs and larva are fixed and sedimented and the faecal debris is separated in a layer between the ether and formal water. Faecal fat is dissolved in the ether.

Requirements:

-Formal water 10% v/v
-Strainer (with small holes 400-450)
-Beaker
-Centrifuge tube
-Slides cover slips
-Screw cap bottle or tube
-Diethyl ether
-Rods
-Pipettes
-Gauze
-Iodine

Method

- Using a rod, emulsify an estimated 1g (pea size) of faeces in about 4mls 10% formal water contained in a screw cap bottle or tube.
- Add further 3-4 mls of 10 % formal water, cap the bottle, and mix well by shaking.
- Sieve the emulsified faeces, collecting the sieved suspension in a beaker.
- Transfer the suspension to a centrifuge tube.
- Add 3- 4 mls diethyl ether.
- Stopper the tube and mix for 1 minute.
- With tissue wrapped around the top of the tube, loosen the stopper.

- Centrifuge immediately at 30000 rpm for 1 minute.
- Using a stick, loosen the layer of faecal debris from the side of the tube and invert the tube to discard the ether, faecal debris and formal water. The sediment will remain.
- Return the tube to its upright position and allow the fluid from the side of the tube to drain to the bottom. Tap the bottom of the tube to resuspend and mix the sediment.
- Transfer the sediment to a slide and cover with a coverslip.
- Examine the preparation microscopically using x10 objective with the condenser iris closed sufficiently to give good contrast.
- Using x40 objective to examine small cysts and eggs.
- To assist in the identification of cysts, run small drop of iodine under the cover glass.

NB:/The number of each species of egg in the entire preparation can be counted. This gives the approximate number per gram of faeces.

MLS 2302: CLINICAL PROTOZOOLOGY

GIEMSA STAINING TECHNIQUE

Staining is a method of imparting colour on cell organelles or their background to enable easy identification of different characteristics of cells and micro-organisms. There are different types of staining methods, but in today's practical, we are going to use giemsa and leishman staining methods.

Materials required

- Prepare thick and thin films
- Giemsa stock solution
- Leishman stain
- Phosphate buffer
- Measuring cylinders
- Oil immersion
- Lancets
- Microscope

GIEMSA STAINING TECHNIQUE (THICK FILMS)

Preparation of stock solution
-Giemsa powder 3.8gm
-Absolute methanol 250ml
-Pure glycerol 250ml
Dissolve the powder in the alcohol in a dry conical flask. Stir using a glass rod until the powder has completely dissolved.
Add the glycerol and store in a labeled reagent bottle.

WORKING SOLUTION

Use 5% or 10% of giemsa stock solution diluted in phosphate buffer pH 7.2.

Preparation of phosphate buffer
Disodium hydrogen phosphate powder (Na_2HPO_4) 0.7gm
Potassium dihydrogen phosphate powder (KH_2PO_4) 0.3gm
Distilled water 1000ml
Weigh the powder or use buffer tablets pH 7.2 and dissolve in distilled water.
TO PREPARE 5% GIEMSA SOLUTION-;
Giemsa stock solution 5ml
Phosphate buffer pH 7.2 95ml
Mix the 2 solutions in a measuring cylinder.
10% Giemsa solution
Giemsa stock solution 10ml
Phosphate buffer Ph 7.2 90ml
Mix the 2 solutions in a measuring cylinder.
METHOD OF STAINING
1. Place the slides on the staining rack.
2. Flood the slides with the diluted giemsa solution. Leave the stain as follows;
   5% giemsa for 20 minutes.
   10 % giemsa for 10 minutes.
3. Wash off excess stain with a thin stream of phosphate buffer. Distilled/ clean water may be used and allow the slides to drain dry and examine them under a microscope.

MLS 2302: CLINICAL PROTOZOOLOGY

LEISHMAN STAINING METHOD (THIN FILM)

1. Put the slides on a staining rack.
2. Pour a little quantity of leishman stain for 3 minutes. Do not leave the stain for too long as it will produce granules.
3. Add twice buffer/distilled water (flood) avoiding overflowing.
4. Allow the diluted stain to act for 10 minutes.
5. Wash in distilled water to produce a rose coloured film.
6. Pour off the water, blot the slide and allow drying in air.
7. Examine using a microscope.

NB; if the slide is slightly stained, remove the immersion oil used with xylene and restain with an equal amount of the stain and buffer/distilled water.

EXERCISE,
With the materials provided, prepare 10 mls of 10% giemsa stain and stain the thick film. Examine the film under the microscope in one field. Draw and label the cells you have seen.
Stain the thin films with the leishman stain provided and draw different cells as you see them.

Stool is collected in sterile containers (polypots) and is immediately processed on arrival at the laboratory. The following is recorded on the container:
1. Patient number.
2. Date of collection.
3. Time the patient passed the stool.
The acceptable size of stool should be pigeon pea size. Examination should be done within 3hrs after collection.

MLS 2302: CLINICAL PROTOZOOLOGY

PRESERVATION AND STORAGE

Stool samples should be preserved, stored and transported to a reference laboratory for confirmation of ova, cysts and helminth larvae. Storage and preservation destroys blood cells, macrophages, motile bacteria and trophzoites of protozoa.

REQUIREMENTS

- Formaldehyde 40%
- Sodium chloride
- Applicator sticks
- Measuring cylinder 100 mls
- Stool sample
- Universal bottles
- Weighing balance
- Gummed labels
- Reagent bottles

- Distilled water

Preparation of 10% formal saline

Formaldehyde 40%	10.0 mls
Sodium chloride	0.85 gms
Tap water	90.0 mls

- Weigh 0.85gm of sodium chloride.
- Dissolve sodium chloride in some amount of tap water.And 10mls of 40% formaldehyde.
- Mix well, and add the remaining water.
- Mix well, label and store at room temperature (ie dissolve 2 in some of the 3,and add 1,and the remaining 3 to make up the volume.)

Method

- Place a portion of the stool sample in a clean universal bottle.
- Add an equal volume of 10% formal saline to the stool sample.
- Mix well with an applicator stick and cap lightly.
- Label the bottle clearly with the patient name,age and date of collection.
- Store the preserved stool sample at room temperature.
- My be stored for up to three months.

Question
State any other method of preserving stool in the laboratory?
Make a report of your report

MLS 2303: CLINICAL HELMINTHOLOGY

FORMAL ETHER SEDIMENTATION TECHNIQUE

Sedimentation techniques have been used as one of the best method in diagnosis of worm infections. The parasites are sedimented by gravity or centrifugal force and therefore a wide range of parasites can be concentrated within minimum damage to the morphology.
Solutions of high specific grafity i.e.solution that is denser than the parasites being concentrated.

Principle:
Faeces are emulsified in formal water, the suspension is strained to remove large faecal particles, ether is added and the mixed suspension is centrifuged. Cysts, oocyst, eggs and larva are fixed and sedimented and the

faecal debris is separated in a layer between the ether and formal water. Faecal fat is dissolved in the ether.

Requirements:

-Formal water 10% v/v
-Strainer (with small holes 400-450)
-Beaker
-Centrifuge tube
-Slides cover slips
-Screw cap bottle or tube
-Diethyl ether
-Rods
-Pipettes
-Gauze
-Iodine

Method

- Using a rod, emulsify an estimated 1g (pea size) of faeces in about 4mls 10% formal water contained in a screw cap bottle or tube.
- Add further 3-4 mls of 10 % formal water, cap the bottle, and mix well by shaking.
- Sieve the emulsified faeces, collecting the sieved suspension in a beaker.
- Transfer the suspension to a centrifuge tube.
- Add 3- 4 mls diethyl ether.
- Stopper the tube and mix for 1 minute.
- With tissue wrapped around the top of the tube, loosen the stopper.
- Centrifuge immediately at 30000 rpm for 1 minute.
- Using a stick, loosen the layer of faecal debris from the side of the tube and invert the tube to discard the ether, faecal debris and formal water. The sediment will remain
- Return the tube to its upright position and allow the fluid from the side of the tube to drain to the bottom. Tap the bottom of the tube to resuspend and mix the sediment.
- Transfer the sediment to a slide and cover with a coverslip.
- Examine the preparation microscopically using x10 objective with the condenser iris closed sufficiently to give good contrast.
- Using x40 objective to examine small cysts and eggs.
- To assist in the identification of cysts, run small drop of iodine under the cover glass.

NB:/The number of each species of egg in the entire preparation can be counted. This gives the approximate number per gram of faeces

MLS 2303: CLINICAL HELMINTHOLOGY

ZINC SULPHATE FLOATATION TECHNIQUE

A zinc sulphate solution is used which has a specific gravity of 1.180- 1.200. Feaces are emulsified in the solution and the suspension is left for the eggs and cysts to float to the surface. They are then collected on a cover glass. This technique is recommended for concentrating the cysts of G. lamblia, E. histolytica, the eggs of T. trichuria, opisthorchis and clonorchis

Requirements
Zinc sulphate solution
Test tube
Strainer

Method

- Fill the tube about one quarter full with the zinc sulphate solution. Add an estimated 1 gram of faeces (2ml if in liquid form). Emulsify the specimen in the solution.
- Fill the tube with the zinc sulphate solution and mix well. Strain the faecal suspension to remove the large debris.
- Return the suspension into the tube. Stand thetube in a completely vertical position in a rack.
- Using a plastic bulb pipette add further solution to ensure the tube is filled to the brim.
- Carefully place a completely clean cover glass on top of the tube, avoid trapping air bubble
- Leave undisturbed for 30-40 minutes to time for the ova and cysts to float
- Carefully lift the cover glass from the tube by a straight pull upwards. Place the cover glass facing downwards on a slide.
- Examine microscopically the entire preparation using the 10X objective with the condenser iris closed sufficiently to give good contrast. Use the 40X objective, and run a drop of iodine under the cover glass to identify the cysts.
- Identify any parasites present.

MLS 2303: CLINICAL HELMINTHOLOGY

GIEMSA STAINING TECHNIQUE

Giemsa stain is an alcohol based rowmanowsky stain that requires dilution in pH7.1-7.2 buffered water before use. It gives the best staining of malaria

parasites in thin films. It also stains thick films well provided they are completely dry, the concentration is low and the staining time is sufficiently long. Less satisfactory results are obtained when the concentration of the giemsa stain is increased to reduce the staining time.
Staining is a method of imparting colour on cell organelles or their background to enable easy identification of different characteristics of cells and micro-organisms.

Materials required

- Prepare thick and thin films
- Giemsa stock solution
- Leishman stain
- Phosphate buffer
- Measuring cylinders
- Oil immersion
- Lancets
- Microscope

GIEMSA STAINING TECHNIQUE (THICK FILMS)

Preparation of stock solution
-Giemsa powder 3.8gm
-Absolute methanol 250ml
-Pure glycerol 250ml
Dissolve the powder in the alcohol in a dry conical flask. Stir using a glass rod until the powder has completely dissolved.
Add the glycerol and store in a labeled reagent bottle.

WORKING SOLUTION

Use 5% or 10% of giemsa stock solution diluted in phosphate buffer pH 7.2.

Preparation of phosphate buffer

Disodium hydrogen phosphate powder (Na_2HPO_4) 0.7gm
Potassium dihydrogen phosphate powder (KH_2PO_4) 0.3gm
Distilled water 1000ml
Weigh the powder or use buffer tablets pH 7.2 and dissolve in distilled water.

To prepare 5% giemsa solution

Giemsa stock solution 5ml
Phosphate buffer pH 7.2 95ml
Mix the 2 solutions in a measuring cylinder.

10% Giemsa solution

Giemsa stock solution 10ml
Phosphate buffer Ph 7.2 90ml
Mix the 2 solutions in a measuring cylinder.

METHOD OF STAINING

1. Place the slides on the staining rack.
2. Flood the slides with the diluted giemsa solution. Leave the stain as follows;

    5% giemsa for 20 minutes.
    10 % giemsa for 10 minutes.

3. Wash off excess stain with a thin stream of phosphate buffer. Distilled/ clean water may be used and allow the slides to drain dry and examine them under a microscope.

MLS 2303: CLINICAL HELMINTHOLOGY

LEISHMAN STAINING TECHNIQUE

1. Put the slides on a staining rack.
2. Pour a little quantity of leishman stain for 3 minutes. Do not leave the stain for too long as it will produce granules.
3. Add twice buffer/distilled water (flood) avoiding overflowing.
4. Allow the diluted stain to act for 10 minutes.
5. Wash in distilled water to produce a rose coloured film.
6. Pour off the water, blot the slide and allow drying in air.
7. Examine using a microscope.

NB; if the slide is slightly stained, remove the immersion oil used with xylene and restain with an equal amount of the stain and buffer/distilled water.

EXERCISE,
With the materials provided, prepare 10 mls of 10% giemsa stain and stain the thick film. Examine the film under the microscope in one field. Draw and label the cells you have seen.
Stain the thin films with the leishman stain provided and draw different cells as you see them.

MLS 2303: CLINICAL HELMINTHOLOGY

PRESERVATION AND STORAGE

Stool samples should be preserved, stored and transported to a reference laboratory for confirmation of ova, cysts and helminth larvae. Storage and preservation destroys blood cells, macrophages, motile bacteria and trophzoites of protozoa.

REQUIREMENTS

- Formaldehyde 40%
- Sodium chloride
- Applicator sticks
- Measuring cylinder 100 mls
- Stool sample
- Universal bottles
- Weighing balance
- Gummed labels
- Reagent bottles
- Distilled water

Preparation of 10% formal saline

Formaldehyde 40%	10.0 mls
Sodium chloride	0.85 gms
Tap water	90.0 mls

- Weigh 0.85gm of sodium chloride.
- Dissolve sodium chloride in some amount of tap water. And 10mls of 40% formaldehyde.
- Mix well, and add the remaining water.
- Mix well, label and store at room temperature (i.e. dissolve 2 in some of the 3, and add 1, and the remaining 3 to make up the volume.)

Method

- Place a portion of the stool sample in a clean universal bottle.
- Add an equal volume of 10% formal saline to the stool sample.
- Mix well with an applicator stick and cap lightly.
- Label the bottle clearly with the patient name, age and date of collection.
- Store the preserved stool sample at room temperature.
- May be stored for up to three months.

Question
State any other method of preserving stool in the laboratory

MLS 2303: CLINICAL HELMINTHOLOGY

LABORATORY DIAGNOSIS OF HELMINTHES

NEMATODES (A. LUMBRICOIDES)

Laboratory diagnosis of A.lumbricoides infection is by:

- Finding A.lumbricoides eggs in faeces, concentration techniques are rarely required.
- Identifying A. lumbricoides worms expelled through the anus or mouth

Requirements

- Stool sample
- Glass slides
- Cover slips
- Microscope

Morphology of fertilized egg

- Yellow brown oval or round, measuring 50-70 µm long by 30-50 µm wide
- Shell is often covered by an uneven albuminous coat (mammilated)
- Contains a central granular mass which is the unsegmented fertilized ovum

Morphology of unfertilized egg

- Is darker in colour and has a thinner wall and more granular albuminous covering
- More elongated than a fertilized egg, measuring about 90 – 45 µm. contains a central mass of large granules

N/B

Decorticated egg

Is an egg that has no albuminous coat, It has a smooth shell and appears pale yellow or colourless.

HOOK WORMS (*N. americanus or A. duodenale*)

In faecal specimens less than 12 hrs old, a hookworm egg has the following appearance:

- It is colourless with a thin shell which appears microscopically as a black line around the ovum
- Oval in shape measuring about 65- 40 µm
- Contains an ovum which appears (usually 4-8 cell stage)

N/B
If the appearance is more than 12 hours old, a larvae maybe seen inside the egg, if the egg is more than 24hrs old, the may hatch and must then be differentiated from a sstrongyloides

MLS 2303: CLINICAL HELMINTHOLOGY

LABORATORY DIAGNOSIS OF HELMINTHES

CESTODES

A concentration technique and the examination of several specimens may be necessary to detect Taenia eggs in faeces.
They are usually few because the eggs are not regularly discharged from the tapeworm in the intestine. Eggs may be concentrated by the formal ether technique

Requirements
- Stool sample
- Glass slides
- Cover slips
- Microscope

Egg of T. saginata and T. solium
- It is round to oval measuring 33-40µm in dimeter
- Embryo is surrounded by a thick brown radially striated wall; hooklets are present in the embryo
- Careful focusing is necessary to see the three pairs of hooklets
- Embryonic hooklets are present in the eggs of both T.saginata and T. solium

MLS 2303: CLINICAL HELMINTHOLOGY

LABORATORY DIAGNOSIS OF HELMINTHES

TREMATODES

Schistosome eggs

When eggs are not found in direct preparations a concentration method should be performed such as the formal ether technique

Morphology of S. mansoni egg

- It is pale yellow, brown, large and oval measuring about 150-60µm
- Has a characteristic side (lateral) spine
- Contains a fully developed miracidium

N/B
Sometimes the spine may appear terminal like that of an S. haematobium egg but if the egg is rolled over by pressing gently on the cover glass, the spine will be seen to be lateral

Morphology of S. haematobium

It is pale yellow, brown, large and oval in shape, measuring about 145-55µm
Has a characteristic small spine at one end (terminal spine)
Contains a fully developed miracidium
A living egg (in fresh unpreserved urine) shows what is called flame cell movement, i.e. flickering of the excretory flame cell

MLS 2303: CLINICAL HELMINTHOLOGY

FIELD STAINING TECHNIQUE

This water based rowmanowsky stain is composed of two solutions, Field stain A and Field stain B. It's buffered to the correct PH and neither solution requires dilution when staining thick films. When staining thin films, field stain B requires dilution. Field stains are more stable, they stain fresh blood well particularly thick films. The rapid technique is ideally suited for staining blood films from waiting outpatients and when reports are required immediately

Materials

- Clean glass slides
- Blood sample
- Immersion oil
- Field stain A & B
- Microscope
- Absolute methanol

METHOD:

(A). Thick Films

Procedure

Prepare thick films as required

Arrange four coupling jars on the bench, label

Stain by dipping the slides in the jars as follows:

- Field stain A-4 seconds
- Clean water- 5 seconds
- Field stain B- 4 seconds
- Clean water- 5 seconds

Allow the slides to air dry and observe using oil immersion

THIN BLOOD FILMS

Reverse staining technique is normally used to stain thin films. The films are first fixed with absolute alcohol then staining with field stain B followed by field stain A.

The technique preserves all blood cells and parasites to be examined

Procedure

Prepare thin films as required

Fill a jar with absolute methanol

Fix the films by dipping in absolute methanol for 1-2 seconds

Stain by dipping the slides in the jar as follows

- Field stain B – 4 seconds
- Clean water - 5 seconds
- Field stain A – 4 seconds
- Clean water - 5 seconds

Allow the slide to drain dry.

Examine using oil emersion objective of the microscope, for trophozoite (ring forms not gametocytes)

“

MLS 2303: HISTOPATHOLOGY AND CYTOLOGY

TEMPORARY TISSUE PREPARATION (FRESH)

INTORDUCTION

There are two types of histological preparations that can be made from histological specimens. These are;

- Fresh preparations
- Fixed preparations

Only fresh preparation will allow the examination of tissue in their living state.

The choice of technique to be used is governed by a number of factors thus;

- What structures and inclusions are to be demonstrated
- The amount and nature of tissue available
- Whether the tissue is fresh or fixed
- The urgency of investigation required

Various methods are used to prepare fresh tissues;

1. Opposition smear

Requirement;

Fresh tissues, microscope, glass slides

Procedure:

1. Crush a selected piece of small soft tissue between two glass slides.

2. Draw the glass slides apart in the opposite directions in a horizontal plane to make a smear on each slide.
3. Examine using a microscope.

2. Macerations

Requirements;
A tissue, nitric acid, dissecting needle, coverslips, and glass slides

Procedure
1. Place the tissue in nitric acid to dissolve the connective tissue in order to isolate the muscle fibres

NB:
The time of isolation varies depending on the type of tissue. Some can take as short as 10 minutes. Others can take as long as 3 days.

2. Place the tissues on a slide then isolate by teasing with a dissecting needle.
3. Wash the tissue with water to remove the chemical mount and then observe.

MLS 2303: HISTOPATHOLOGY AND CYTOLOGY

FIXATION

A fixative is a chemical preservative used in fixation. It is usually in a gaseous or solution form. In most cases it is in solution form.
The aim of a fixative is to;
- Preserve the cell and tissue shape.
- Preserve the cell and tissue shape, their structure relationship and chemical constituents, after death.

Preservation should be such that, the fixed tissues resembles as closely as possible the form which it had during life. Most fixatives in histology are made up of two or more simple fixatives each with its special effects on the cell or tissue in order to produce their combined effects of their individual action. Those single fixatives are called simple fixatives and the product of two or more single fixatives is known as compound fixatives.

Preparation of fixatives:
1. 10% formal saline. Duration of fixation 24-48hrs and more.
   - Formaldehyde -10ml
   - Sodium Chloride -0.85gm
   - Tap Water -90ml

Dissolve sodium chloride in some amount of tap water.

Add 10ml of 40% formaldehyde. Mix well and add the remaining water. Mix well, label and store at room temperature.

2. Carnoy's fluid- cytological compound fixative.

- Absolute alcohol -60ml
- Chloroform -30ml
- Glacial acetic acid -10ml.

Mix the ingredients above well, label and store.

Fixation

Trim the provided tissue using a sharp scalpel blade on a trimming board to obtain small sizes of tissues.

Using a pair of forceps, transfer the tissues into specimen bottles containing the fixatives prepared. Allow the tissues to fix for a minimum of 24hrs.

Questions

1. What are the factors affecting the rate of fixation?
2. What are the advantages and disadvantages of 10% formal saline as a fixative?

MLS 2303: HISTOPATHOLOGY AND CYTOLOGY

DECALCIFICATION

INTRODUCTION

Decalcification is the removal of calcium from the bone to facilitate section cutting. A tissue must be thoroughly fixed in order to be to withstand the harsh effects of decalcifying solutions. For general purposes, formaldehyde is suitable but for bone-marrow, zenker formal should be used,

Note;

If an acid decalcifying solution is used, don't use a fixative containing an acid. If a large piece of bone contains a pulpy centre, it can be frozen at -20C before fixing then cut to the desired size with a fine hacksaw. This prevent the soft tissues being dragged out, thus giving a false histological picture. After this fix and proceed as with an ordinary bone.

Requirements

- Fixed bone/ teeth
- Mineral acid (formic acid)

Procedure

Formic acid is prepared as follows;

- Formic acid (S.G. 1.2) -5-10ml
- Distilled water -100ml

(Decalcification period: 2-7 days)

1. Fix the tissues thoroughly.
2. Immerse the tissues into the formic acid.
3. Leave the tissues to decalcify for 2 days.
4. Test the end point of decalcification using the chemical method.

Technique:

1. Put 5ml of decalcifying fluid in a test-tube and drop in a piece of blue litmus paper (the paper must turn red).
2. Add strong ammonia drop by drop until the litmus paper turns blue to show that the decalcifying solution is alkaline.
3. Turbidity at this stage shows that the fluid contains plenty of calcium.
4. If the solution remains clear, add 1ml of 5% ammonium oxalate and let it stand for 30 min.
5. Turbidity at this stage shows that, there is little calcium in the decalcifying solution. No turbidity shows that the decalcifying solution is free of calcium and therefore decalcification is complete.

Question:

What are the criteria of a good decalcifying solution?

MLS 2303: HISTOPATHOLOGY AND CYTOLOGY

TISSUE PROCESSING

INTRODUCTION:

The examination of tissues with a microscope requires very thin slices of tissues that can transmit light. The preparation of the thin sections is called microtomy. Before such tissues are sliced or sectioned, they must undergo preparatory treatment through various reagents entailing infiltration of the tissues with suitable embedding medium which provides internal and external support to the tissues.

The embedding medium must be of good consistency for easy microtomy. This preparatory treatment tissue before sectioning is

called tissue processing. Processing method employed is named after the embedding medium used. There are many embedding media used in histology, each with its special advantages and limitations.
The recommended tissues processing method is that which uses the paraffin wax.

Materials & Equipments:

- Fixed tissues
- Ball string
- Molten paraffin wax
- Scalpel blade
- Paraffin wax oven
- Various grades of alcohols
- Clearing agents
- Cotton gauze
- Trimming mats
- Labels and pencils

Procedure

1. Fixed tissues are trimmed using a sharp scalpel blade preferably 3-5mm cube sizes.
2. The tissues are placed at the centre of two layered cotton gauze accompanied by a label written in pencil.
3. Tissues are tied tightly using a ball string and then washed in running water for 12hrs.
4. Dehydration is done using ascending grades of alcohols i.e. 50%, 70%,80%, 90% and two changes of absolute alcohols. Immerse the tissues for 1 ½ hrs into each reagents.
5. Clearing of alcohols by immersing tissues into two changes of clearing agents e.g. methyl benzoate, for 1 ½ hrs.
6. Infiltrate the tissues in paraffin wax (molten) maintained at 2-3 degrees Celsius above the melting point of wax. This is done in the paraffin wax oven.
7. Untie the tissues and embed them in fresh pure molten paraffin wax using paper boats. Leave the moulds to solidify on a flat bench.

Question

During tissue infiltration, what are the dangers of using high temperatures?

MLS 2303: HISTOPATHOLOGY AND CYTOLOGY

BLOCKING AND SECTIONING

INTORDUCTION

After embedding, the solidified mould of wax containing the tissue should be attached to a wooden block.

This wooden block is clamped on a microtome block holding during sectioning. The microscope is designed to facilitate the study of animal tissues by transmitted light and for this purpose the tissue must be sliced into thin lamellae or sections. These are cut at a pre-determined thickness which depends on the character of the tissue. Uniform thickness can only be assured by using a machine known as a microtome. Microtomes of various designs are made for use with different tissues-supporting media. For preparing paraffin sections, the rocker, rotary and sliding patterns are normally used.

Materials and Requirements:

- Rotary microtome & knife
- Embedded tissue blocks
- Wooden blocks
- Warm water bath
- Hot plate/ Bunsen burner
- Glass slides
- Forceps camel hair brush
- Egg albumen adhesive
- Diamond pens
- 20% alcohol
- Wooden-handled spatula

Method

1. Heat a wooden handled spatula over a Bunsen burner & hold it to the surface of the block holder.
2. Place the paraffin block on the hot spatula to melt the wax.
3. After a few seconds, withdraw the spatula and press firmly the paraffin wax block onto the holder.
4. Seal all the edges with the hot spatula.
5. Fix the block holder in position on the microtome.
6. Turn back the feed mechanism.
7. Insert a suitable knife in the microtome & secure it with the tightening screws.
8. Move the block holder forward until the paraffin block is almost touching the knife edge. The block must be accurately trimmed before it is attached to the block holders.
9. Orientate the block holder so that the surface to be cut and the lower edge of the block are parallel to the knife edge at the moment of the impact.
10. Check all the tightening screws on the microtome.
11. Set the thickness gauge at 15 microns and trim the block until the whole surface is being cut. The block is now ready for sectioning.
12. Set the gauge to the required thickness and operate the microtome until complete are cut maintaining a regular cutting rhythm.
    If the upper and the lower edge of the block are parallel to the knife, the sections will form a ribbon.
13. Support the free end of a ribbon with a camel hair brush.

Floating out sections on water,

1. Set the temperature of water bath at 6-10 degrees Celsius. Lower than the melting point of wax
2. Flood glass slide with 20% alcohol.
3. Lower the sections, shinny surface downwards onto the alcohol.
4. Lower the slides gently into water so that, the sections will be carried by convection currents set up by the alcohol. All creases in the tissues should disappear within few seconds.
5. Insert one of the prepared slides (smeared with a drop of egg albumen adhesive & inscribed with diamond pen)
6. Withdraw the slide keeping it in a vertical position and the section will adhere to its surface.
7. Position the section at the centre of the slide with a mounted needle. Allow the excess water to drain off.
8. Transfer the slides to a thermo-statistically controlled hot plate or an incubator for several hours, to ensure that the sections are thoroughly dried before being stained.

Question:

What are some of the faults encountered in cutting paraffin sections and their remedies?

MLS 2303: HISTOPATHOLOGY AND CYTOLOGY

STAINING OF TISSUES AND COVERSLIPPING

INTRODUCTION:

Although many basic dyes have been used as nuclear stains, haematoxylin still remain the standard nuclear stain for histological studies.
Haematoxylin by itself is a very weak dye and is of no value in micro-technique.
It is always used in conjunction with mordant that causes it to act as very strong basic dye. The mordant may be used separately from the haematoxylin solution (indirect staining haematoxylins) or mixed with haematoxylin solution (direct staining haematoxylins).
Indirect staining haematoxylins require that, the tissues be first exposed to mordant. These are typically heavy mental ions (usually iron) that bind to the negatively charged sites on the tissues. Excess, unbound mordant is washed out and then the section is exposed the haematoxylin which binds to the mordant and provides the coloration.
Direct staining haematoxylin stains incorporate the mordant into the stain formula and hence stain directly without any preliminary mordant treatment. They are much faster than iron haematoxylin and are the routine stains used in histological staining.

Materials:

- Staining jars and baskets
- 1% acid alcohol
- Sections mounted on glass slides
- Various grades of alcohol
- Xylene
- Eosin
- DPX mountant

Procedure:

1. Dewax sections with 2 changes of xylene each 5 minutes.
2. Hydrate as follows; 2 changes of absolute alcohol each 5 minutes.
3. 90% alcohol 2 min, 80% alcohol 2 min, 70% alcohol 2 min, tap water 5 min.
4. Stain in haematoxylin stain 10-30 minutes.
5. Wash thoroughly in running tap water for 5 minutes.
6. Differentiate in 1% acid alcohol (3 quick dips).
7. Blue sections in running tap water 5-10 minutes.
8. Counterstain with 1% Eosin for 3 min.
9. Dehydrate sections starting with 70%, 80%, 90% for 1 min each then two changes of absolute alcohol for 5 min.
10. Clear in 2 changes of xylene, 5 minutes each.
11. Mount in DPX mountant and cover slip.

12. Leave the slides on a flat bench to dry at room temperature.
13. Examine under a microscope starting with low power objective and then higher power for more details.

Question:

Draw and label the structures you observe.

MLS 2303: HISTOPATHOLOGY AND CYTOLOGY

CYTOLOGY –
Is the branch of biology concerned with the study of the structure and function of cell as individual units. It supplements histology; cytology was for a long time concerned with the microscopic observation of stained dead cells and the correlation of such observations with known physiological phenomena. But recently, new procedures have been introduced by which the living cell can be observed and studied.

METHYLENE BLUE STAIN FOR CYTOLOGICAL SMEARS (BUCCAL SMEAR)

Materials
Glass slides, Droppers, Compound microscope, Applicator sticks, Methylene blue, and cover slips.

Method
Rinse your mouth with clean water and then lightly scrape the inside of your cheek (oral mucosa) using the applicator stick and make a thin smear on the slide. Allow the slide to air dry. Add one drop of methylene blue stain to your smear and cover it with a cover slip.
Observe under a microscope (mucosal cells), draw and label.

1. Urine specimens

Cytological examination of urine is important in the detection of cancer of the
urinary tract, prostate and renal parenchyma.

Method

Centrifuge urine sample provided at 200RPM for 5-10min
Discard the supernatant.
Make the smear from the small volume deposit of urine at the bottom of tube.
Air dry the smear and stain with methylene blue.
Examine and draw the cells seen.
NB: Methylene blue being a basic dye stains the acidic nuclear blue.

BACTERIA (ACID FAST BACILLI)

Histological bacteria are demonstrated to determine whether they are gram positive or gram negative for the bacilli, it is to determine whether they are ethanol fast or acid and ethanol fast. The common bacteria that are demonstrated are colonies of coccid in necrotic tissue and bacilli, leprosy bacilli and tubercle bacilli.

Materials; mounted sections on glass slides carbol fuchsin sulphuric acid, methylene blue.

Staining technique.

1. Bring sections to water after Dewaxing.
2. Cover the section with a piece of filter paper.
3. Flood the section with filtered carbol fuchsin for 15min.
4. Remove the filter paper and wash well with tap water
5. Decolorize with 20% sulphuric acid for 3 mins or 1% acid alcohol (3 quick dips)
6. See if the sections are pale pink.
7. Repeat step 5and 6 until the desired picture is obtained.
8. Counter stain with methylene blue for 2-3 minutes.
9. Wash in water
10. Dehydrate in 50% 70% 80% 90% & 2 changes of absolute alcohol
11. Clear in 2 changes of Xylene
12. Mount in DPX.

Results

Nuclei –Blue

Acid fast bacilli- bright red

Red blood cells –pale pink.

MLS 2303: HISTOPATHOLOGY AND CYTOLOGY

Cervical smears

This is used for the diagnostic of cancer of the cervix,
Cells are collected from the squamo-columnas junction where most of these lesions originate. The cervix is visualized using an unlumbricated speculum; the longer projection on the Ayre spatula is placed in the cervical os and rotated through 360 degrees. The cellular material is quickly smeared onto labeled glass slides and fixed immediately. The smears should then be stained with papanicolaou technique.

Materials: cercal smears permanent stained smears.

Microscopy.
Method; Examine the slides first using the low power and then high power objective.
Draw and label the cells seen.

MLS 2303: HISTOPATHOLOGY AND CYTOLOGY

Papanicolaou stain

This is the most popular cytological stain because it gives excellent nuclear detail, makes the cytoplasm translucent and being colorful, it helps determine the age of the cells.

Solutions 1. Harris Haematoxylin.
2. Orage G solution (O G 6)
0.5% Orange G in 95% ethanol 100mls phosphotongstic acid - 0.015gms.
3. Eosin in Azure 50 (EA50)
0.5% light green SF (yellowish) in 95% ethanol 45mls
0.5% Bismarck brown in 95% ethanol -10mls
0.5% Eosin y in 95% ethanol -45mls

Phosphotongstic acid -0.2gm
Mix well filter and store in a tightly capped brown bottle

Principle of the stain. Haematoxylin stains the nuclei blue by dye –lake formation. The eosin azure solution being acidic stains the cytoplasm which is basic so that the eosin has affinity for the young endocervical cell. Oranges are also being an acidic dye has an affinity for the cytoplasm of the oldest superficial cells.

Staining Technique.
1. Fix the smears in the appropriate fixation.
2. Hydrate smears by passing them through ethanol grades 80%, 70% and then 50%.
3. Rinse in distilled water- 10 dips.
4. Stain in Harris Haematoxylin -4min.
5. Rinse in tap water.
6. Differentiate in 1% acid alcohol 10 dips.
7. Rinse & blue in tap water – 10 dip.
8. Rinse & blue in 95% ethanol – 10 dips.
9. Stain in O. G. 6 for 2min.

10. Rinse in 95% ethanol – 10 dips.
11. Stain in E.A. 50- 5 mins.
12. Rinse in 95% ethanol – 10 dips.
13. Dehydrate in changes of absolute ethanol – 10 dips each.
14. Clear in 3 changes of Xylene – 10 dips each.
15. Mount in DPX.