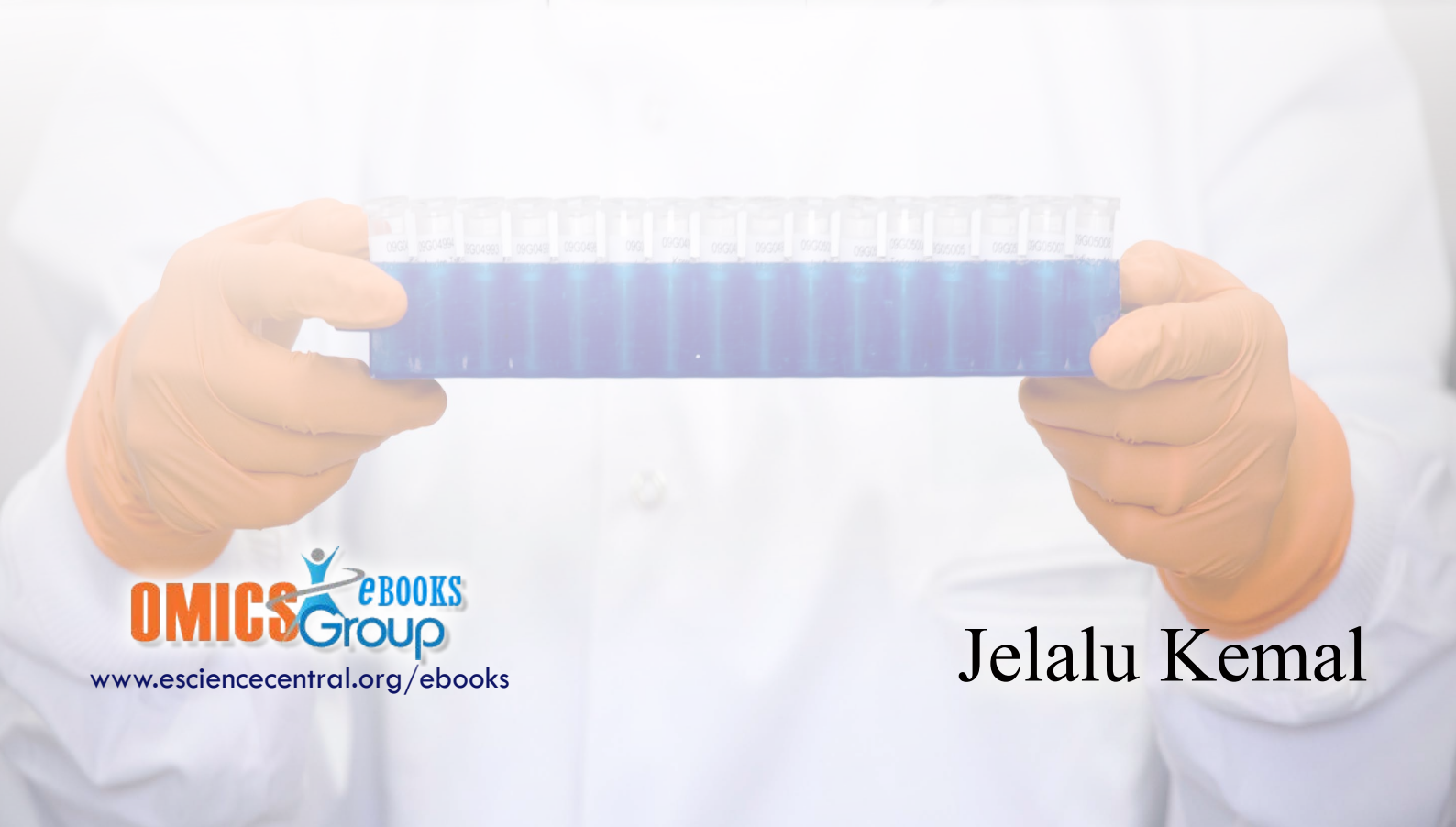


Laboratory Manual and Review on Clinical Pathology



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Abstract

Clinical pathology is a subspecialty of pathology that deals with the use of laboratory methods (clinical chemistry, microbiology, hematology and emerging subspecialties such as molecular diagnostics) for the diagnosis and treatment of disease. Hematology studies the blood and blood-forming tissues to evaluate presence of disease and assist in therapeutic interventions as clinically indicated. Clinical chemistry (also known as chemical pathology and clinical biochemistry) is the area of clinical pathology that is generally concerned with analysis of bodily fluids. Some of the objectives of this manual are to identify the most important hematological and functional pathological tests of vet importance, to diagnose different animal diseases by confirming the pathological causes that constraint live stock production and to have knowledge more about clinical pathology. Part one discusses about hematology which includes equipments and reagents, blood collection sites and procedures, preparation method for working solution, staining methods (staining procedures), hemoglobin determination, hematocrit determination (PCV), total RBC count, total WBC count, differential leukocyte count, determination of ESR, coagulation time determination, bleeding time, calculating red blood cell indices and blood group and Rh factor determination. Part two deals with function tests which includes determination of Aspartate Amino Transferase (AST) and Glutamic Oxalacetate Transaminase (GOT), determination of Alkaline Phosphatase (ALP), determination of creatinine, total protein determination, urea determination, total and direct bilirubin determination, enzymatic kinetic colorimeter test, liver function test, kidney function test, rumen function test and pancreatic function test. In general, the outline of this laboratory manual deals with the basic hematological procedures and clinical chemistry analysis using which is meant for students use. All of the procedures described in this manual are classical methods that are routinely executed in a standard clinical pathology laboratory.

Keywords: Clinical chemistry; Clinical pathology; Hematology; Laboratory tests; Manual

Introduction

Clinical pathology is one of the two major divisions of pathology, the other being anatomical pathology. Clinical pathology is itself divided in to subspecialties, the main ones being clinical hematology/blood banking, clinical chemistry, hematopathology and clinical microbiology and emerging subspecialties such as molecular diagnostics. Hematology studies the blood and blood-forming tissues to evaluate presence of disease and assist in therapeutic interventions as clinically indicated. Clinical chemistry (also known as chemical pathology and clinical biochemistry) is the area of clinical pathology that is generally concerned with analysis of bodily fluids.

Blood parameters play a critical role in diagnosis, assessing progression, and in the characterization of disease and phenotypes in clinical and research situations. The accuracy and reliability of the whole blood parameter analysis depends on identification and control or elimination of variables that may affect these results. Different blood collection and handling strategies represent source of variability that can be controlled in many instances.

Basic hematological procedures such as the complete blood count are frequently conducted to help physicians and veterinarians arrive at a diagnosis and prognosis, also to evaluate treatment. Hematology testing includes, but is not limited to: Hematology Sample Processing, Hematology Research Services, Hematology Automated Testing, Lamellar Body Counts, Microscopic Cellular Analysis, Body Fluid Analysis and Specialized Stains.

The outline of this laboratory manual deals with the basic hematological procedures and clinical chemistry analysis which is meant for students use. The study of hematology begins with proper sample collection and handling. Part one deals with hematology containing different sections. Part two covers clinical chemistry including functional tests. All of the procedures described in this manual are classical methods that are routinely performed in a standard clinical pathology laboratory. Detail procedures including preparation of reagents, solutions, stains and buffers are given. Calculations of blood indices and chemical analysis are also discussed in part one and part two respectively.

Objectives of Clinical Pathology Practical Course

At the end of Clinical Pathology practical course the students will be able to:

- Identify the most important hematological and functional pathological tests of vet importance
- Diagnose different animal diseases by confirming the pathological causes that constraint live stock production in general
- Have knowledge more about clinical pathology
- Write lab report pertaining the tests carried out

Part One: Hematology

Equipments and reagents required for hematological blood examination.

- Hematocrit centrifuge
- Compound microscope
- Sahlis instrument
- Capillary tube
- Hematocrit reader
- Distilled water
- Haemocytometer pipette
- Haemocytometer counting chamber
- WBC diluting fluid
- RBC diluting fluid
- Slides, cover slip, special cover slip
- ESR stand, ESR tube
- Filter paper, blood lancets, lead pencils, marker
- Giemsa stain, Wright's stain
- Vacutainer tubes(different type), vacutainer needles, syringes, needle holder
- Gloves
- Blood samples
- Staining racks and others

Blood Collection

Site: The following lists of veins are the most appropriate blood collection sites from different species of veterinary importance

Jagular vein- the most commonly used site in the horse, cattle, sheep, goat, camel and large wild mammals: used occasionally in small animals. Vacutainer tube, vacutainer needle, syringe, needle, needle holder, and disinfectant should be used for blood sample.

Procedure

- Placement of the thumb of the left hand in the jagular furrow to occlude and anchor the jagular vein, while manipulating the syringe and needle with the right hand
- Clipping the site of sampling especially in long haired animals is recommended
- The veins are more clearly out lined when the site is rubbed with alcohol

Cephalic vein - The most commonly used site for collection of small amount of blood in the dog. By constricting the area on the dorsal aspects of the fore limb at the level of the elbow which can be raised beginning just above the carpal vein.

Ear vein- can be used in small dog, pig, cat and small lab animals (small dog, rabbit, guinea pig, and monkey).

- A marginal vein on the dorsal side of the ear is selected
- Remove the hair by shaving, clipping, or other method
- Swab the skin with alcohol or other
- Place the left index finger under the ear at the point of applying stylet or syringe
- Gentle aspiration is used when using a syringe in small animals like rabbit, to avoid collapse of the vein

Toe or Toe nail – can be used in small dog, puppy, guinea pig etc

- Clip the hair away
- Disinfect the capillary bed of the nail
- Cut in to just short of the base of the nail
- Take 20-40 drops as well as 80 drops (4ml) of blood

Tail – can be used in pig, cattle, sheep, rat, and mouse

- Veinipuncture –of the coccygeal vein on the ventral side
- Amputation – commonest method used in the rat and mouse. A small transverse incision with a razor blade produce drops of blood

Heart – may be used in animals like bird, fish, and others

Femoral or Tibial vessels – used in dog, cat, small mammals, rat etc

Mammary vein – used for dairy cattle

The vein appears at the anterior border of the mammary gland lateral from the linea Alba and runs forward passing through a foramen in the abdominal wall posterior to ribs.

Anterior vena cava – used for the pig

A needle ($4\frac{1}{2}$ – 6 in. and 17 to 20 gauge needle) is inserted just anterior and slightly lateral to the cariniform cartilage and a line from the cartilage to the base of the ear.

Retro orbital Venus plexus – used for the rat, mouse, guinea pig

- A best method for obtaining a large quantity of blood (1ml) in mouse
- This technique is reported to be less traumatic than others

Wing vein or comb – used for birds

- After the feathers in the axillary region are plucked, the alar vein is seen running from beneath the pectoral muscle then along the ventral surface of the humerus

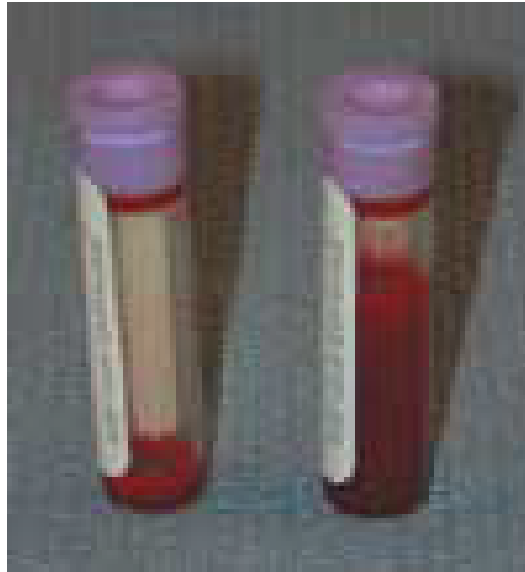


Figure 1: EDTA tubes should be filled to their labelled capacity. Short filled tube has an excessive amount of EDTA relative to the blood volume. Short filled tubes cause a false increase in total plasma protein content and a false decrease in PCV and RBC count.

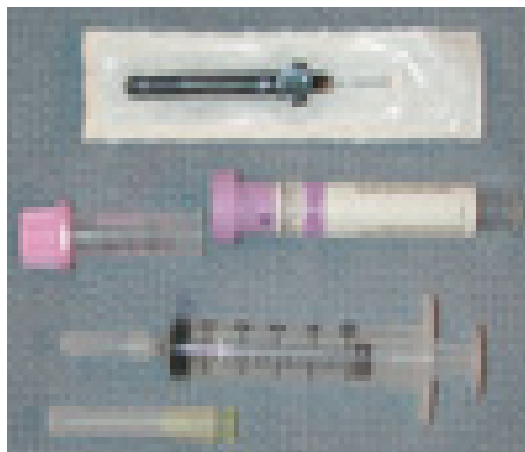


Figure 2: Collection materials for hematological assessment include syringe, needles, and purple topped collection tubes that contain EDTA.

In most animals up to 0.5 ml/kg blood collection has no any adverse effect. The total volume of blood collection differs in different animals.

Vacutainer tubes

- a. Red-stopper tubes – are for tests requiring clotted blood
- b. Lavender stopper tubes – contain EDTA in concentrated liquid or desiccated powder form
- c. Green stopper tubes – contain heparin and are used for blood gases, PH, (CO_2 , O_2)....
- d. Gray stopper tubes – contain oxalates, fluorides, or citrates
- e. Yellow stopper tubes – available with Acid Citrate Dextrose (ACD) solution or physiological saline solution

Anticoagulants

The most important anticoagulants that can be used during blood collection with different mode of action are listed below.

Anticoagulant / product	Mode of action	Amount required	Advantage	Disadvantage
EDTA/Ethylene diamine tetra acetic acid	Form insoluble Ca salts	10-20mg (1ml of 1 % solution dried at room temp. or at incubator	Recommended for routine hematological procedures, preserve cellular elements better	May shrinks cell because Na salt is less soluble
Heparin	Antithrombin and antithromboplastin	1-2mg (0.2ml of 1% solution)	Less effect on RBC hemolysis Used for blood gas analysis	May cause clumping of WBC, unsuitable for smears, as it interferes with stain ability of WBC expensive
Na citrate	Combine with Ca to form an insoluble Ca salt	10-20mg (1ml)	Can be used for blood transfusion	Interferes with many chemical tests, shrink cells
Potassium oxalate	Units with Ca to form insoluble calcium oxalate	20mg (2 drops of 20% solution dried in incubator	Very soluble	Causes shrinkage, it increase the volume of blood
Sodium oxalate	Units with Ca to form insoluble calcium oxalate	20mg	Used mainly for prothrombin time	Same as potassium oxalate

Table 1: Commonly used anticoagulants, their mode of action and dose required.

Staining Dyes

Wright's stain: Forms of dye available

- Dry powder as bulk and as preweighed vials, Capsule
- Readymade solution. Most convenient, but often deteriorated from long storage

Preparation of stain

Conventional method

- Place 0.5gm of dry Wright's stain powder in a mortar
- Add 300ml of absolute methyl alcohol
- Grind the mixture and pour in dark bottle
- Shake the bottle each day for about two weeks, then filter
- Aging of the stain will take 2 to 4 before it will be satisfactory for use

Accelerated method

- Place 0.3gm of dry Wright's stain powder in a mortar and over lay with 3ml of glycerol, grind thoroughly
- Rinse with 100ml of absolute methyl alcohol and place in dark container
- Mix with magnetic stirrer for about 1 wk without heat
- Filter before use

Giemsa Stain

General consideration

- Giemsa stain has various azure compounds with eosin and methylene blue
- It is an excellent stain for blood parasites and for inclusion bodies
- It stains red granules well but neutrophilic granules and erythrocytes are poorly stained
- Commercial stock solution are recommended for purchase and are stable indefinitely

Preparation method for working solution: Take one part of giemsa stock solution and nine part distilled water and mix before one to two hours before use.

Leishmen's stain

Preparation

- Grind 0.15gm of Leishmen's stain powder with small amounts of absolute methyl alcohol until an even suspension is obtained. A total of 100ml methanol is added to produce complete solution
- Pour in to a dark bottle and age for a few weeks prior to use

Wright's-Giemsa stain (modified Wright's stain)

Preparation of stain

- 500mg of dry Wright's stain powder and 50mg of Giemsa stain powder are ground in a mortar with 100ml of absolute methyl alcohol (acetone free)
- Allow to stand for 24-48 hrs before using
- Keep well stoppered to prevent evaporation of alcohol or absorption of water vapor

Commercial Wright's – Giemsa stain are available

- Wright – Leishman stain
- May – Grunwald –Giemsa stain
- Methylene blue stain
- Field's stain
- Prussian blue stain and others

Buffered distilled water

Preparation: 5.47g monobasic potassium phosphate and 3.8g of dibasic sodium phosphate with 1000ml of distilled water

Methods of Staining (staining procedure)

Wright's stain

- Cover the dried blood smear completely with Wright's stain and allow to stand for 1 to 3 minute
- Add an equal amount of buffered distilled water or neutral water (pH 6.6 to 6.8 for most animals blood)
- Blow it gently and allow the mixture to stand for 3 to 5 minutes. A metallic scum should appears green
- Float off the metallic scum with a stream of water from a wash bottle or from the top. Use distilled water if tap water is too alkaline. Don't pour the stain off the slide before washing, as this will result in a precipitate forming on the slide

- Wipe the stain from the back of the slide with cleaning tissue
- Stand the slide on end, wave gently in the air, or blot gently with bibulous paper to dry
- Examine the preparation by placing a drop of oil on a slide
- If a permanent preparation is desired, the oil can be dissolved with xylene and a mounting medium like Canada balsam is applied.

Advantage: It conserves stain, Methyl alcohol, and time

Disadvantage: More precipitate is likely to be present

Cell	Cytoplasm	Chromatin
Erythrocytes	Yellow to pinkish	Purple
Lymphocytes	Blue	Purplish red
Monocytes	Light blue	Purple
Eosinophils	Yellow to brownish red rods	Light purple
Basophils	Dark purple granules	
Hetrophils/Neutrophils	Yellow to brownish red rods	Light purple
Thrombocytes	Grey-blue	Purple

Table 2: Staining characteristics.

Giemsa stain method

- Place dried blood smear in a coplin jar containing absolute methyl alcohol for about 3 minute to fix the smear
- Drain off the alcohol and allow the slide to dry
- Transfer the slide to a second coplin jar containing fresh stain and allow staining for 16 to 60 minute. 60 minute staining period is used when blood parasite is suspected. 12 to 18 hrs for inclusion bodies
- Wash the stain with running/tap water, dry and examine under oil immersion microscope

Leishman's stain method

- Flood the air dried blood film with Leishman's stain and leave for 1 to 2 minute to fix
- Dilute the stain on the smear with double the volume of buffered distilled water and stain for 5 to 15 minute
- Blow on the mixture gently
- Wash with distilled water until the film has a pinkish tinge
- Wipe the back of the stain/the slide and allow to dry in upright position

Smear Preparation

- Wet smear
- Thin smear
- Thick smear

Procedures for each preparation

Wet blood smear/film preparation

- A drop of blood is placed at the centre of a clean slide
- Cover with a clean, dry cover slip
- Examine the film under the microscope ($40\times$ objective)

The method does not require staining. It is rapid and simple to perform.

Extracellular blood parasites such as Trypanosomes and Microfilaria of filarial nematodes can be diagnosed. Note that you can see only the movement of Trypanosomes

Thin blood smears preparation

It can be made by spreading a drop of blood evenly across a clean grease free slide using a smooth edged spreader.

- Make a drop of blood on one end of glass slide
- Place the end of second glass slide /spreader slide / against the surface of the first slide, holding at an angle of 30-45 degrees
- Draw the spreader slide gently into the drop of blood and when the blood has along 2/3 of width of the spreader slide by capillary action, push the spreader slide forward with a steady even motion
- Dry by waving rapidly in the air

Thick bloods smear preparation

- A large drop of blood is put at the centre of a clean dry slide
- The drop is spread with an applicator stick, needle or corner of another slide to cover an area of $\frac{1}{2}$ an inch square
- The smear is thoroughly dried in a horizontal position so that the blood could not ooze to one edge to the film and protected from dust, insects and direct sunlight.

Causes of inadequately stained blood smears

Analysis of the poor results will determine what modification of technique is required to correct the problem

Entire smear is too blue - granules of neutrophil over stained and larger than normal, and granules of the eosinophil is deep gray.

- Buffered water, wash water or stain too alkaline
- Excessive thickness of the smear
- Alkaline residue to the slide
- Insufficient washing
- Prolonged staining before diluting with buffered water

Nuclei of leukocytes are pale blue, and the erythrocytes and granules of eosinophils are usually red.

- Buffered water, wash water or stain too acid
- Acid residue on slide

Entire smear has a pale stain

- Under staining
- Weak stain
- Excessive washing or allowing water to stand on slide
- Using warm or hot water for washing slide

Variation in staining on different areas of the smear

- Buffered water un evenly applied and not thoroughly mixed with Wright's stain
- Acid or alkaline residue on the slide
- Water not properly drained from slide after washing

Precipitated stain

- Lack of through washing
- Precipitate in Wright's stain not properly filtered
- Evaporation of alcoholic stain

Hemoglobin Determination

Objective: To determine the amount of hemoglobin present in 100 ml of blood of a given sample.

Significance

- It serves as an index of blood condition of the animal.
- If the hemoglobin [Hb] content falls below the normal levels, it indicates anemia, or pregnancy (physiological).

c. If it increases to the normal value, it indicates polycythemia, decrease in O₂ supply, heart disease, emphysema etc.

Method: Acid hematin method

Requirements: Sahlis instrument, blood sample

Procedure

- Take 0.1N HCl (1%) into central graduated tube up to mark 2.
- Suck the blood exactly up to mark 20 (20 µl) with the help of sahlis pipette.
- Transfer the blood from pipette to central graduated tube of the hemometer.
- Mix it well with the help of stirrer or rod and allow it to react for two minute.
- Make up with distilled water by adding drop by drop until the color matches with the Standard comparator tube and mix well.
- When the color matches take out and record the values on the side as gm/100ml and or in percentage.
- Repeat 5 to 6 times and take the average value

Normal value: Bovine	8 - 15gm/dl (11)	Equine	11 - 19gm/dl (14)
Ovine	9 - 15gm/dl (11.5)	Feline	8 - 15gm/dl (12)
Caprine:	8-14gm/dl (11)	Canine	12 - 18gm/dl (15)

Hematocrit Determination (PCV)

Aim: To determine the hematocrit value for a given blood sample.

Principle: Blood compartment is separated into three parts using capillary tube in a hematocrit centrifuge.

Method: Wintrobe hematocrite method

Significance

- Packed Cell Volume (PCV) = erythrocyte mass; anemia when PCV falls dawn.
- Buffy coat; white to gray layer above PCV. It will give number of WBC (0.5mm to 1.5mm). Leukopenia or leukocytosis.
- Plasma content: usually about 55%, Yellowish in color. Degree of yellowness indicates icterus (jaundice).

Requirements: Hematocrit tube, hematocrite centrifuge, hematocrit reader and sealer.

Procedure

- The blood is filled in to a micro hematocrit tube (3/4th) and seals it with sealer.
- Centrifuge the filled hematocrit tube in a hematocrite centrifuge at 2000 rpm for 4-5 minutes.
- Read the value (the tube) with hematocrit reader and record the result.

Normal value

- Bovine 37 - 55 % (45%)
- Ovine 24 - 50 % (38%)
- Caprine 24 - 50 % (40%)
- Equine 32 - 55 % (42%)
- Swine 32 - 50 % (42%)

Total Count of RBC

Objective: To enumerate the total count of RBC/cumm of a given blood sample.

Method: Hemocytometry method

Significance

- It performs some functions such as transportation of O₂ and CO₂
- A decrease in RBC accounts for less hemoglobin i.e., anemia
- An increase in RBC is referred as Polycythemia

Requirements: Hemocytometer, cover slip, microscope, RBC diluting fluid, Haeyem's solution or Physiological saline 0.85% NaCl.

Procedure

- Take the blood in to RBC pipette up to 0.5 marks
- Immediately draw the RBC diluting fluid up to mark 101.

- Rotate the pipette between thumb and other fingers with finger eight (8) movements. This gives a dilution of 1:200.
- Clean the counting chamber of hemocytometer and cover slip
- Place the cover slip in position over the counting chamber by gentle pressure
- Expel a drop of blood on to the counting chamber by holding the pipette at an angle of 45°.
- Allow the hemocytometer for 2-3 min to settle down the RBC in counting chamber
- Counting: Counting rules
 - Count less than 40 × microscope objective
 - Count cells touching the left and top side lines.
 - Don't count cells touching the bottom right side lines.
 - Count first left to right direction, then to vise verse.

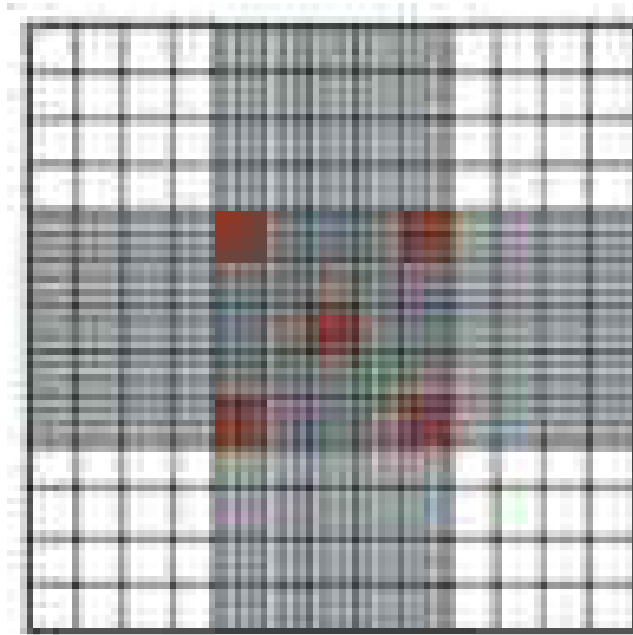


Figure 3: Hemocytometer grid lines. Erythrocyte and leukocyte count. Red = zones to be counted under high power for erythrocytes. White = zones to be counted under low power for leukocytes.

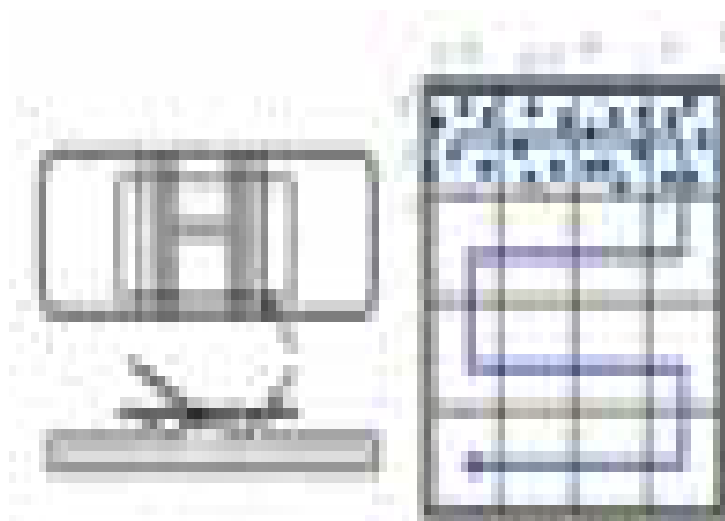


Figure 4: The hemocytometer and the direction in which the count should be made.

Calculation

Volume of one small square = $1/20\text{mm} \times 1/20\text{mm} \times 1/10\text{mm} = 1/4000\text{mm}^3$

Volume of 80 small square = $80 \times 1/4000\text{mm}^3 = 1/50\text{mm}^3$

Total number of RBC = Cells counted (N)/Volume of all squares \times dilution factor

Total RBC = N (cell counted)/1/50mm³ \times 1/200 = N \times 10,000

Total Count of WBC (White Blood Cells)

Objective: To enumerate the total number of WBC/cumm of blood.

Method: Hemocytometer method

Significance

The normal WBC performs some important physiological functions. The chief function of it is imparting immunity to the body. Therefore, the decrease or increase in circulating WBC indicates physiological condition i.e., increase in WBC indicates inflammation where as decrease in WBC may indicate AIDS.

Requirements: Hemocytometer, cover slip, microscope, WBC diluting fluid, or 1% HCl (1% Glacial Acetic Acid) i.e., 1ml GAA + 1 ml MB + 100ml dist.H₂O.

Procedure

- Draw the blood in to WBC pipette up to 0.5 marks.
- Immediately draw the WBC diluting fluid up to 11 marks.
- Rotate the pipette between thumbs and finger horizontally. This will give you a dilution of 1:20.
- Clean the counting chamber of hematometer and cover slip.
- Place the cover slip on the counting chamber with gentle pressure.
- Expel the fluid in the pipette by an angle of 45°.
- Allow the hemocytometer for 2 minute to settle down the WBC.
- Count the WBC in the 4 large squares in the corners of counting chamber (16 small squares).

Counting

Count cells touching the left and top side lines

Don't count cells touching the right and bottom side lines

avoid duplication of cells

Calculation

Total WBC count = Cells counted \times 50

Area of one smallest square = $1/4 \times 1/4 = 1/16\text{mm}^2$

Depth of the counting chamber = 1/10mm

Volume of one smallest square = $1/10\text{mm} \times 1/16\text{mm}^2 = 1/160\text{mm}^3$

4 corners \times 16 ss = 64 ss \times $1/160\text{mm}^3 = 64/160\text{mm}^3 = 0.4\text{mm}^3$

Total volume = 64 ss \times $1/160\text{mm}^3 = 64/160\text{mm}^3 = 0.4\text{mm}^3$

Dilution = 1:20

Therefore, Total WBC = cell counted/dilution \times volume

= cell counted/1:20 \times 0.4

= cell counted \times 20/0.4

= cell counted \times 50

Differential Leukocyte Count

Aim: To enumerate the different types of leucocytes namely neutrophil, eosinophil, basophil, monocyte, and lymphocyte.

Method: Blood film (smear) with Wright's or Giemsa stain

Significance

- They are responsible for the defense of the organism
- They are 5000-7000mm³
- Leucocytes divide in to granulocyte and agranulocyte

Neutrophils

- Very active in phagocytizing bacteria

- Found in highly in pus
- Died after phagocytosing pathogens
- High during inflammation

Eosinophils

Attack parasites and phagocyte antigen-antibody complexes

Basophils

- Secrete anticoagulant and vasodilatory substances (histamines, serotonin)
- Their chief function is secreting substances

Lymphocytes

The main constituents of immune system defense against viruses, bacteria, fungi and protista.

Monocytes

Precursors of macrophages. Have intense secretory activities (lysosomes, interferons)

Requirements

Clean glass slide, sterile needle, microscope, stains solution, oil immersion

Protocol

Making the smear

- Place a small drop of blood near an end of the slide
- Bring another slide in contact with the drop and allow distributing at an angle of 30-40 degrees
- Push to the left in a smooth and quick motion
- Dry the slide with air

Fixing - Dip the smear in a vessel containing 95% containing ethyl or methyl alcohol for 3-5 minutes

Staining

- Stain the smear with Giemsa or Wright's stain for 16-60 or 1-3 minutes respectively
- Rinse the slide with distilled water at room temperature
- Drain off the water and leave the slide to dry

Cover slipping

Place a drop of Canada balsam on the smear and then mount the cover slip

Observation

- examine under dry objective or oil immersion
- count about 100-200 cells and take average

Interpretation

Neutrophils

- Very tiny light staining granules
- Multi-lobed nucleus connected by thin strands of nuclear material
- Are most numerous, normally they account 50-70%
- If the count exceeds this amount, the cause is usually due to an acute infection (appendicitis, small pox, rheumatic fever)
- If the count is considerably less, it may be due to a viral infection (influenza, hepatitis, rubella)

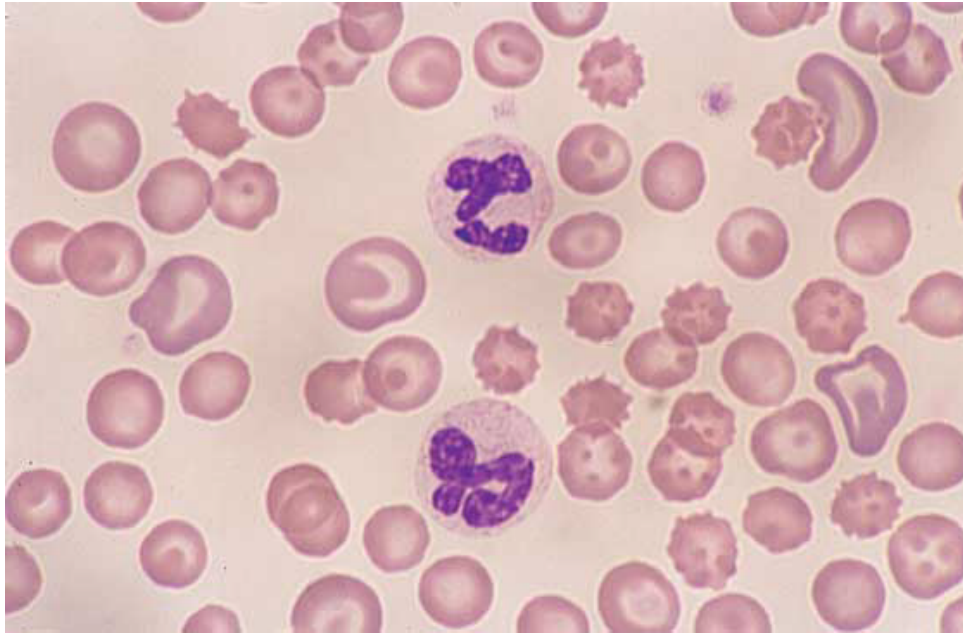


Figure 5: Normal canine neutrophils. Both neutrophils have a lobulated nucleus in a light pink finely granulated cytoplasm.

Eosinophils

- Has large granules (acidophilic), pink red
- The nucleus often has two lobes connected by a band of nuclear material, looks like telephone receiver which contains digestive enzymes against parasites.

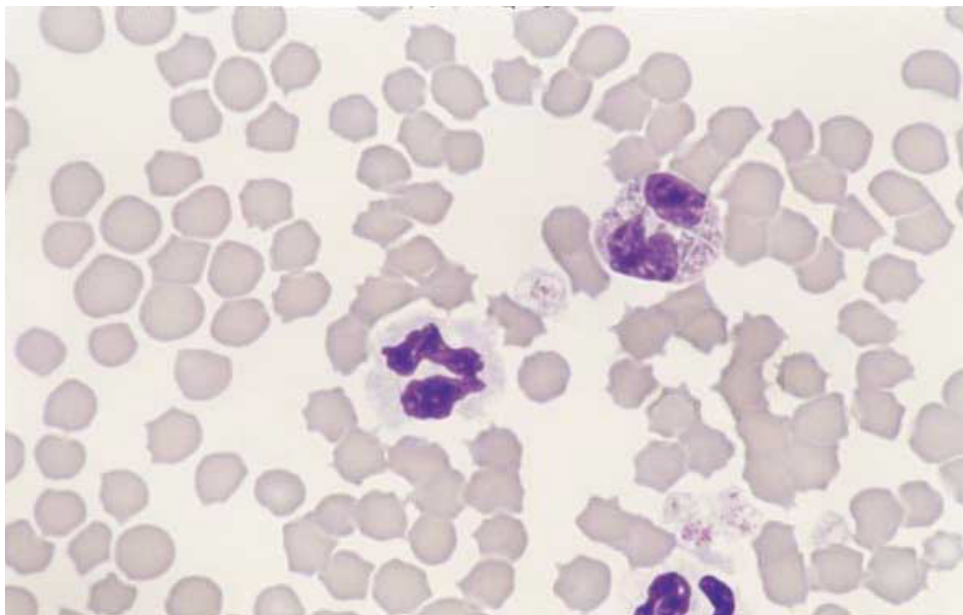


Figure 6: Feline eosinophils. Eosinophil (top) has granules that are pale pink and rod-shaped.

Lymphocytes

- An agranulocyte cell with very clear cytoplasm
- Very large nucleus which fills almost the cell with leaving thin rim of cytoplasm (Figure 5).
- Smaller than the three granulocytes (N,E,B)
- Play an important role in our immune response
- The T-lymphocytes act against virus infected cells and tumor cells
- The B-lymphocytes produce antibodies
- 2nd numerous leukocyte, accounting for 25-35% of the counted in differential WBC count
- When number of this cells exceeds the normal value, one would suspect parasites
- Patients with AIDS keep a careful watch on their T-cell level

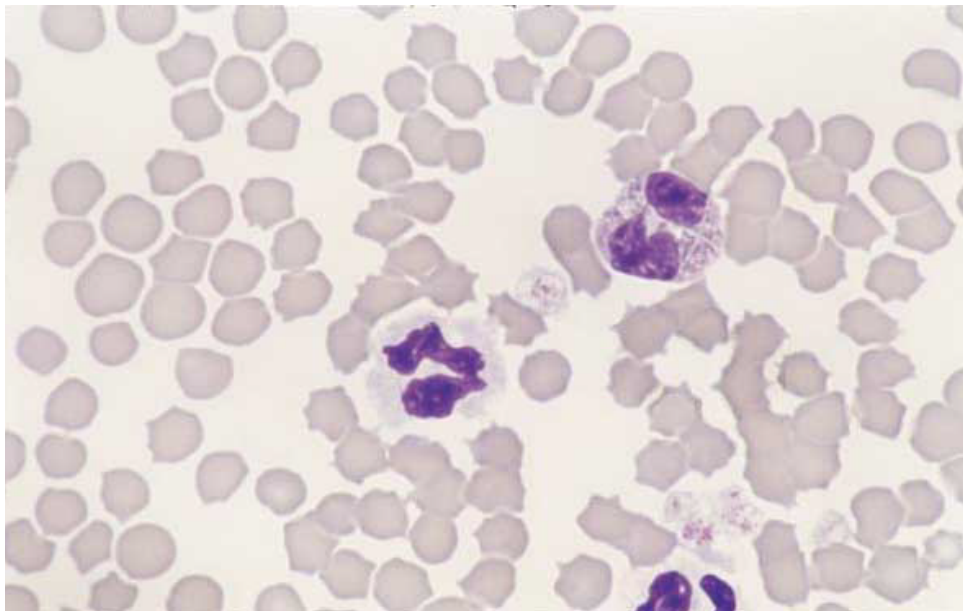


Figure 7: Normal lymphocytes. Lymphocytes in the dog and cat are the same size or smaller than a neutrophil. Feline lymphocytes are similar to those in the dog (A and C 100x, B 120x).

Monocytes

- The largest of leucocytes and are agranular
- “U” or kidney bean shaped nucleus
- Abundant cytoplasm and light blue
- Account for 3-9% of the total leukocyte
- People with malaria, endocarditis, typhoid fever, and rocky mountain spotted fever, monocyte increases in number

Normal value

Neutrophil = 50-70%

Eosinophil = 2-4%

Basophil = 0-1%

Lymphocytes = 20-40%

Monocytes = 3-8%

Determination of Erythrocyte Sedimentation Rate (ESR)

Purpose: To determine the ESR of a given blood sample

Principle: The distance (in mm) that the erythrocyte fall during a given period of time when blood to which anticoagulant has been added in a tube placed in a vertical position

Significance

- It is not a specific test, but reflects change in plasma protein accompanying most of acute and chronic infection
- Some pathological condition causes rouleaux formation
- The greater the ESR reading, the more the severity of pathological condition
- During TB and rheumatic disease ESR increases

Method: Westergrens method

Requirements: ESR stand, ESR tube, blood sample

Protocol

- Take the anticoagulant blood in to ESR tubes exactly up to ‘0’ mark.
- Place the tube vertically (upright position) in ESR stand.
- Take reading after 5min as ‘zero’ hour reading and again note the reading after 1 hour and 2 hours.

Normal value: 0-mm at 1hr, 2-3mm at 24hrs, but if above this value at 24 hrs, it indicates some pathological defect

Horse	10-30 min
Cattle	8-24hr

Sheep	24hr
Goat	24hr
Pig	8hr
Dog	1hr, because of large sized erythrocyte
Cat	1hr
Man	1hr

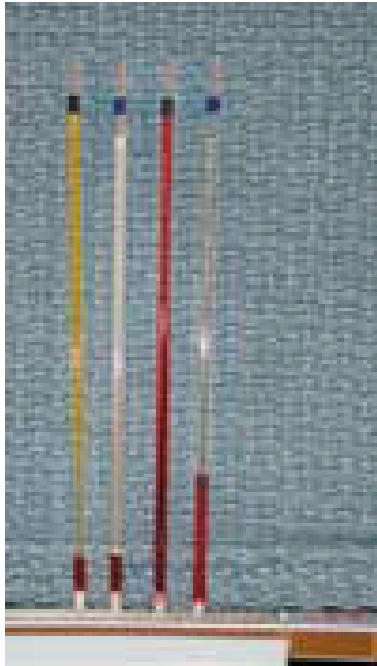


Figure 8: Erythrocyte sedimentation rate.

Coagulation Time Determination (Whole Blood Clotting Time)

Lee-white method

- Obtain at least 3ml of blood in a plastic syringe by careful vein puncture (start a stop watch)
- Place 1ml of blood into each of the three tubes
- Place the test tube in a water bath at 37°C
- After 2 minutes one of the three test tubes is tipped gently at one minute interval
- Test the third test tube in the same manner
- The time elapsed between the first appearance of the blood in the syringe and clot formation in the third tube is clotting time

Capillary tube method

Capillary tube, filter paper, clock watch should be used as requirements

- A skin puncture is made and wiping away the first drop, fill a special capillary tube with blood noting the time when the blood first appeared
- Holding the tube between the thumb and index finger of both hands, gently break the tube every second until a strand of thread fibrin is seen extending across the gap between the ends of the tube
- The interval between the appearance of the blood and the appearance of the fibrin stand is the coagulation time

Interpretation

Normal value

- Lee- white method in glass tube --- 3-12 minutes
- Capillary tube method---3-15 minute in horse and cattle---1-5 minutes other animals

Prolonged

- Deficiency in coagulation factors
- Vitamin K deficiency
- Thrombocytopenia
- The presence of circulating anticoagulants

Species	Time in minutes
Bovine	3-5
Ovine	1-6
Caprine	2.5-11.5
Equine	3-15
Porcine	4
Canine	3-4
Feline	5

Table 3: Normal values of whole blood clotting time in various species of domestic animals (capillary tube method).

Bleeding Time

Determination of bleeding time is a simple and sometimes useful tool for evaluating the efficiency of the capillary – platelet aspect of homeostasis

Purpose: To determine the bleeding time

Method: Dukes method

Significance

- The study helps in diagnosis, treatment, and study of hemorrhagic diseases
- The prolonged time indicates coagulation defect

Requirements: Blood lancet, filter paper, clock (stop clock)

Protocol

- Make a moderately small, deep puncture in clean, sterile blood lancet or sterile needle, and note the time when blood first appears (nose is preferable)
- Remove the drops of blood with filter paper every 30 second being careful not to touch the skin. The use of highly absorbent paper such as cleaning tissue tends to prolong bleeding time due to more effective removal of surface blood
- Note the end point, when blood no longer appears from the puncture site

Interpretation

Normal values: 1- 5 min in domestic animals

Prolonged due to

- vascular lesions
- platelet defect
- severe liver disease, uremia
- anticoagulant drug administration

Calculating Red Blood Cell Indices

Objectives

- To be able to calculate MCV, MCH, and MCHC from red blood cell count, hematocrit and hemoglobin concentration
- To compare with normal ranges of the blood indices of different domestic animals

Significance

Here one can classify red blood cells as normo, hypo, and hyperchromic anemia. Apart from these facts, the size of red blood cell as normo, micro, and macrocytosis can be classified.

Materials

The following formulas depict method of calculating the blood indices

$$MCV = \frac{Ht\% \times 10}{TRBC} = 90 fl$$

- MCV indicates a change in the size of a single RBC
- Normocytosis means normal size
- Microcytosis indicates smaller size due to iron deficiency etc and thus lower hemoglobin concentration
- Macrocytosis indicates larger size and is usually caused by vit B12 and folic acid deficiencies

$$MCH = \frac{Hg(g / dl) \times 10}{RBCNo(10^6 ul) 30} = 30 pg$$

- MCH is mostly associated with hemoglobin concentration
- Decreased MCH causes hypochromic anemia because of a deficiency of iron in the diet
- Increased MCH may be caused during hemolysis where extracellular hemoglobin level increases

$$MCHC = \frac{Hb(g/dl) \times 100}{Hct(\%) } = 33\% pg$$

• MCHC is the most accurate of the indices, because it doesn't require RBC counts. decreased value indicates iron deficiency and reticulocytosis and increased value may be due to hemolysis

Discussion

Considering the size, the calculated RBC indices indicate what?

What differentiates MCHC from other two RBC indices?

Blood group and Rh-factor determination

Objective: To be able to perform ABO and Rh- blood grouping

The following table shows characteristics of the ABO blood group

Blood groups	Antigens (on blood cells)	Antibodies (in plasma/serum)
A	A	Anti - B
B	B	Anti - A
O	No antigen	Anti - A and anti - B
AB	A & B	No antibody

Table 4: Blood group characteristics.

Materials

- Marked plastic plate or slide
- Sterile lancet
- Uncoagulated blood
- Sticks for mixing
- Antiserums

Method

- Place one drop anti - A anti body (blue) on the first bore of the plastic plate
- Place one drop anti - B anti body (yellow) on the second bore of the plastic plate
- Add a drop of blood in to each of the above anti-serums
- Using a separate clean stick for each bore, mix the blood and the antiserums
- Tilt the plate gently from side to side to enhance agglutination
- Observe for agglutination reactions following few minutes
- Similar steps follow for Rh blood, except you use anti - D antibody

Interpretation

Agglutination in a specific well therefore indicates the presence of specific antigen. If no agglutination is observed in anti - A and anti - B antibodies, then blood group is O. if agglutination is observed in both A & B antibodies, the blood group is AB.

For Rh factor, agglutination with anti -D serum indicates Rh+, and no agglutination indicates Rh- blood.

Results

1. Identify if which blood groups and Rh factors show agglutination reaction with the corresponding antibodies, Mark (+) for agglutination and (-) for no agglutination reactions (Table 5).

Blood groups						
Serum	A	B	AB	O	Rh+	Rh-
Anti -A	+	-	+	-	+	-
Anti -B	-	+	+	-	+	-
Anti -D					+	-

Table 5: Blood groups and Rh factor characteristics.

2. Determine your Owen blood group and decide which member of your colleagues could donate or receive blood to each other

No	Your blood group	You can donate blood to	You can receive blood from	Remark
1	E.g. A	B ,AB	B ,O	

2	B	A, AB	A, O	
3	O	A, B, AB	O, A, B	
4	AB	-		

Discussion

Answer the following questions based on your results

What is the cause of transfusion incompatibilities in ABO blood groups?

What blood type is a universal donor and why is it said a universal donor?

State two differences between the ABO and Rh blood type?

What would happen if Rh- mother bears a child from Rh- father?

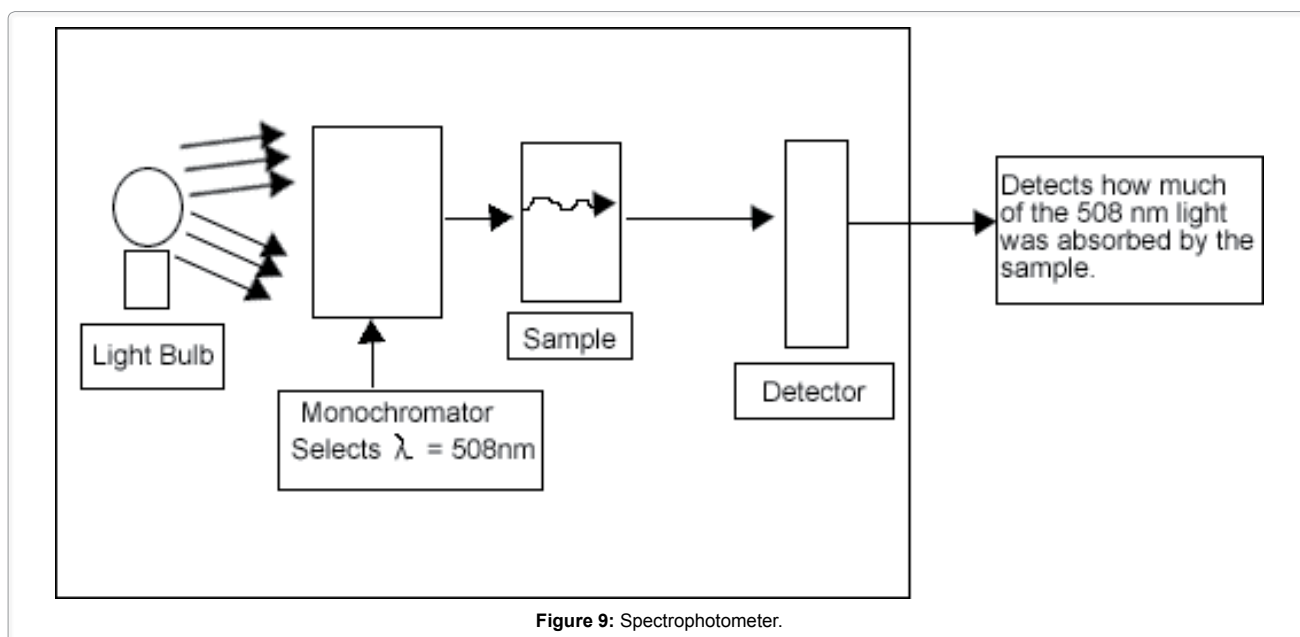
What pathological consequences would arise if Rh+ father marries Rh- mother?

Part Two: Function Tests

The spectrophotometer

It is an instrument used to measure the amount of light absorbed by the solution. It includes

- Light source- gives steady white light
- Prisms- splits white light to visible colors (red, orange, yellow, green, violet)
- Monochromator (wavelength selector)- system for isolating radiant energy of a desired wave length and excluding others
- Cuvate – contains sample solution
- Detector (photoelectric tube)-converts the transmitted light energy into an equivalent amount of electrical energy.
- Meter (recording device)-displays the amount of transmitted light or provides numerical display of absorbance



Beer lambert's law

States that the light passing through colored medium is absorbed in direct proportion to the amount of the colored substance in the light path

$A = abC$ where A – Absorbance

a – molar absorptivity (constants)

b – Length of light path (constants)

C – Concentration

Molar absorptivity (a) – is the absorbance of molar concentration of substance with path length of 1cm determined at specific wave length under defined conditions of solvent , PH and temperature

$A = abC$, $a = A/ bC$

For standard solution and sample solution a - is the same

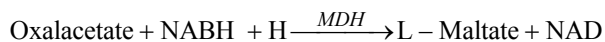
$$\frac{A_{\text{standard}}}{b_{\text{standard}} \times C_{\text{standard}}} = \frac{A_{\text{sample}}}{b_{\text{sample}} \times C_{\text{sample}}}$$

For both solutions b (light path) is the same, so that

$$C_{\text{sample}} = \frac{A_{\text{sample}} \times C_{\text{standard}}}{A_{\text{standard}}}$$

Determination of Aspartate Amino Transferase (AST) and Glutamic Oxalacetate Transaminase (GOT)

Principle: Aspartate Amino Transferase catalyzes the transfer of the amino group from aspartate to oxoglutarate with the formation of glutamate and oxalacetate.



Reagent composition

R1 – AST substrate: TRIS buffer 121mmol, L – aspartate 362mmol/l

Maltate dehydrogenase > 460ul, lactate dehydrogenase > 600ul

R2 – AST coenzyme: NADH

Reagent preparation

Working reagent – mix 4ml of R1 + 1ml of R2 (stable for 4 weeks at +2 to 8°C)

Samples

Serum and EDTA or heparinized plasma

Procedure

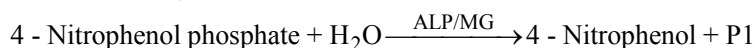
- Pre incubate working reagent , samples and controls to reaction temperatures
- Set the photometer to “0” absorbance with distilled water
- Pipette in a cuvette

Reaction To	37°	30°
Working reagent	1ml	1ml
Sample or control	50ul	100ul

- Mix gently by inversion, insert the cuvette in to the cell holder and start stop watch
- Incubate for 1 minute and record the initial absorbance
- Repeat the absorbance reading exactly after 1, 2, and 3 minutes
- Calculate the difference between absorbance
- Calculate the mean of the results to obtain the average change in absorbance per minute ($\Delta A \text{ min}$)
- Calculation: $\Delta A \text{ min} \times 3333$ (37°) or $\Delta A \text{ min} \times 1746$ (30°)

Determination of Alkaline Phosphatase (ALP)

Principle: Alkaline Phosphatase catalyzes the hydrolysis of 4 – nitrophenyl phosphate (4 – NPA) with the formation of free 4 – nitrophenol and inorganic phosphate



Reagent composition

R1 – ALP buffer: DEA buffer 1.25mol/l, MgCl 0.6mmol/l, biocides

R2 – ALP substrate: 4 – NPP 50mmol/l, biocides

Reagent preparation

Working reagent – mix 4ml of R1 + 1ml of R2 (stable for 4wks at +2 to 8°)

Samples

Serum or heparinized plasma, or EDTA, oxalates, citrates inhibit enzyme

Procedures

- Preincubate working reagent , sample and controls to reaction temperatures
- Set the photometer to ‘0’ absorbance with distilled water
- Pipette in a cuvette

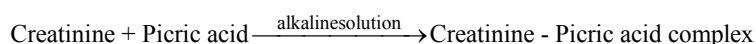
Reaction temperature	37°C
Working reagent	1ml (ALP)
Sample control	20ul

- Mix gently by inversion , insert the cuvette in to the cell holder and start stop watch
- Incubate for 1min and record the initial absorbance
- Repeat the absorbance reading exactly after 1, 2, and 3 minutes
- Calculate the difference between absorbances
- Calculate the mean of the results to obtain the average change in absorbance per minute ($\Delta A/\text{min}$)

Calculation: $\Delta A/\text{min} \times 2746$

Determination of Creatinine

Principle: Creatinine in alkaline medium react with picrate ions to form yellow orange complex whose color intensity is measured at 492nm



Reagent composition

R1 (picric acid) – 35mmol/l

R2 (NaOH) – 0.32mmol

R4 (Creatinine standard) – 2mg/dl

Reagent preparation

Mix equal volume of R1 and R2: Stable for 10 days at 4 to 8° or 1 day at + 20 to 25°

Specimen

Serum, plasma, urine (diluted with distilled water)

Procedure

	Standard	Sample
Working solution	1000ul	1000ul (1ml)
Standard	100ul	-
Sample	-	100ul

Mix and pour into a cuvette after exactly 20seconds read A1 of sample and standard exactly 80sec after first reading, read A2 of sample and standard.

$$C_{\text{test}} = \frac{\Delta A_{\text{test}} \times C_{\text{std}}}{\Delta A_{\text{std}}}$$

Normal values (mg/dl)	Dog	Cow	Horse	Sheep	Goat	Man
	0.1 – 0.8	0.6 – 1.8	0.9 – 2.0	0.9 -2.0	0.7 – 1.5	0.7 -1.2

Table 6: Normal values of creatinine.

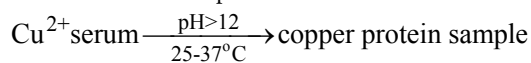
Conversion of mg/100ml into mmol/l and vice versa

$$\text{Mmol/l} = \frac{\text{mg/100ml} \times 10}{\text{Molecular wt}}$$

$$\text{Mg/100ml} = \frac{\text{mmol/l} \times \text{molecular wt}}{10}$$

Total Protein Determination

Principle: In the biuret reaction, a chelate is formed between the Cu^{2+} ion and the peptide bonds in protein in alkaline solution to form a violet colored complex whose absorbance is measured to the concentration of protein in the sample



Reagent composition

R1: Biuret reagent (cupric sulphate 6mmol/l, sodium – potassium tartrate 21mmol, potassium iodide 6mmol, sodium hydroxide 0.75mol/l, CAL protein standard [Bovine Serum Albumin 7g/dl (70g/l)])

Samples

Serum, EDTA or heparinized plasma and exudates. Total protein is stable in serum for one week at room temperature, for at least 1 month refrigerated at 2 – 8°C, for up to 2 months at -20°C

Interferences: Grossly haemolytic or lipemic samples result in positive interference

Materials required

- Photometer or colorimeter capable of measuring absorbance at $540 \pm 20\text{nm}$
- Constant temperature incubator set at 37°C
- Pipettes to measure reagent and samples

Procedure

- Pipette in to labeled tubes

Tubes	Blank	Sample	Standard
Biuret	1ml	1ml	1ml
Sample	-	20ul	-
Standard	-	-	20ul

- Mix and incubate the tubes for 10 min at 37°C
- Read the absorbance (A) of the sample and standard 540nm against the reagent blank

Calculation

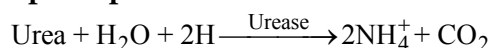
$$\frac{A_{\text{sample}}}{A_{\text{standard}}} \times C_{\text{standard}} = \text{g/dl total protein}$$

If the results are to expressed as SI units apply $\text{g/dl} \times \text{g/l}$

Urea Determination

Use: enzymatic in vitro test for the quantitative determination of urea in serum, plasma and urine samples

Test principles



The ammonium ions formed react with the salicylate and hydrochloride to give a green dye

Reagent composition

- R1: Phosphate buffer PH 6.7----- 60mmol/l
EDTA-----1.5mmol/l
Sodium salicylate----- 60mmol/l
Sodium nitroprusside ----- 5.2mmol/l
R2: Urease----- >5000u/ml
R3: Sodium hydrochloride ----- 18mmol/l
Sodium hydroxide ----- 450mmol/l
R4: Urea ----- 50mg/dl (8.325mmol/l)

Preparation and stability

Working reagent: Add one vial R2 to bottle of R1. It is stable up to 4 weeks at 2 to 8°C , 6days at 20 to 25°C .

R3 and R4 are ready for use

For photometers, which need a minimum of 2ml, dilute R3 by 4 volume of distilled water, eg 10ml of R3 and 40ml of distilled water. The dilute reagent is stable for 6 months at 2 to 8°C .

Specimen

Serum, or heparinized plasma, urine diluted with 1:10 part with distilled water

Limitations

Interference, Icterus, hemolysis, Lipemia

Testing procedure

- Controls = 0.9% NaCl
Wave length = 580 – 600nm
Temperature = 20/25/ 37°C
Cuvette = 1cm light path
Zero adjustment = against reagent blank

	Reagent blank	Standard	Sample
Working solution	1000ul	1000ul	1000ul
R4(standard)	-	10ul	-
Sample	-	-	10
Incubate at +37°C for 5min , or for 10min at 20/25°C , then add			
R3 or diluted reagent /R3 (1:5)	200ul	200ul	200ul
	1000ul	1000ul	1000ul
Mix, incubate at +37°C for 5min or for 10min at 20/25°C and read absorbance against reagent blank (ΔA)			

Calculation

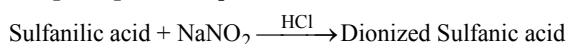
$$\text{Urea conc. (mg/dl)} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times \text{standard conc.}$$

Whereas, R4 /standard concentration = 50mg/dl (8.325mmol/l)

Total and Direct Bilirubin Determination

Use: Quantitative determination of direct bilirubin and total bilirubin in serum or heparinized/EDTA plasma

Test principle: In the presence of caffeine accelerator, total bilirubin couples with sulphonlic acid to form a red azobilirubin dye



Reagents:

R1: Sulfonlic acid -----29mmol/l

Hcl -----0.17mmol/l

R2: Sodium nitrate -----25mmol/l

R3: Caffeine -----0.26mol/l

Sodium benzoate -----0.52mol/l

R4: Tartrate -----0.93mol/l

NaOH -----1.9mol/l

Preparation

R1 ready for use

Specimen: Fresh serum, heparinized /EDTA plasma. Hemolysis interferes with result

Testing procedure: materials – working solution above, calibrators, controls (0.9% Nacl solution)

Wave length – Hg 578nm

Temperature – 20 to 25°C

Cuvette – 1cm

Zero adjustment – against sample blank

	Sample blank	Sample
R1	200ul	200ul
R2	-	50ul
R3	1000ul	1000ul
Sample	200ul	200ul
Mix and incubate at room temperature for 10 to 60 minutes, add		
R4	1000ul	1000ul
Mix and incubate at room temperature for 5 – 30 minutes, read absorbance of sample		

Calculation

$$\text{With calibrator Bilurubin} = \frac{\text{ÅÅ sample}}{\text{ÅÅ calibrator}} * \text{calibrator conc.}$$

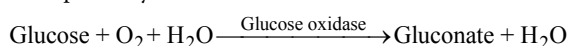
With factors concentration in mg/dl = 10.8 * Δ Abilirubin total

N.B –Direct bilirubin determination should have its own procedure and calculation findings

Glucose

Enzymatic Kinetic Colorimeter Test (GOD – PAP)

Principle: Enzymatic colorimeter test on basis of Trinder-Reaction



Reagent

R1: buffered enzyme reagent

Phosphate buffer, PH 7.5 ----- 0.5mol/l

Phenol ----- 7.5mmol/l

GOD (Glucose oxidase) ----- 12000u/l

POD ----- 660u/l

4-amino-antipyrine-----0.4 mmol / l

R4: Standard

Glucose -----100mg/dl (5.55mmol/lit)

Preparation: the reagent is ready for use

Sample: Serum or heparinized plasma

Procedure

Wave length -----Hg 546nm (492-550nm)

Temperature ----- +25 (+30) +37°C

Cuvette ----- 1cm light path

Zero adjustment ----- Reagent blank

	Blank	Standard	Sample
Working reagent	1000ul	1000ul	1000ul
Standard/R4	-	10ul	-
Sample	-	-	10ul

Mix, measure and incubate at 37°C for 15 minute or 30min at +25°C with in 60min, read absorbance

Calculation

$$\text{Glucose (mg/dl)} = \frac{\text{AA sample}}{\text{AA standard}} \times \text{standard conc.}$$

Standard conc: 100mg/dl (5.56mmol/l)

Liver Function Test

Vander Bight's test

Principle of the test

Direct reaction

• Bilirubin – in the serum from obstructive jaundice reacts immediately with Ehrlich's Diazo reagent, while serum with hemolytic jaundice requires the addition of alcohol and a longer time interval for the reaction

• Measurement of direct 1-min bilirubin demonstrates the glucuronide compound while 30-min bilirubin represents total bilirubin. The quantity of free (indirect reacting) bilirubin is the difference between the total (30min) and direct (1min) values

• Qualitative method

Direct reaction

- Place 1ml of non hemolyzed serum from a fasting animal in a graduated centrifuge tube
- Allow 0.5ml of Ehrlich's Diazo reagent must be prepared each day by mixing 10ml of solution A with 0.3ml solution B below (keep in brown bottle in refrigerator)

Solution A

Solution B

Sulfanilic acid -----1gm

Sodium nitrite -----0.5ml

Conc. HCl acid -----15ml

Dist H2O ----- 100ml

Dist H2O-----1000ml

• The contact zone between the serum and the reagent is examined against a good back ground for the development of a reddish purple ring in 30sec

Indirect reaction

• After the determination of the direct reaction shake the tube containing the serum and diazo reagent and add 3ml of 95% alcohol and mix

• If a definite pinkish color fails to appear in 15min and only a white turbidity occur, the reaction is recorded as Negative. If a color already presents from a positive direct test depends up on the addition of alcohol, the reaction is recorded as positive for the indirect test.

Wallace-Diamond Test

Principle: Depends up on the reaction of urobilinogen and other chromagens with Ehrlich's benzaldehyde reagent to form a red color

Reagents: Ehrlich's benzaldehyde reagent

Para – dimethyl amino benzaldehyde – 2gm

2% aqueous solution of conc. HCl – 100ml

Procedure

- Take 5ml of urine in a test tube, add 0.5ml of Ehrlich's benzaldehyde reagent
- It is essential that the urine be fresh since urobilinogen exposure to light and air is changed into urobilin, which doesn't react
- If large amount of bile are present in equal parts of urine and 110% aqueous solution of barium chloride should be mixed and filter to remove all precipitate as bilirubin interferences with reading the result of the urobilinogen reaction
- Allow the tube to stand for 5 minutes and observe for pink to red color development by viewing lengthwise through the tube against a white background

Result

Normal – pink to light red color

No urobilinogen – absent of pink or red color

Increased urobilinogen – cherry red color

Kidney Function Test

Gross examination of urine /physical examination/

Urine volume: This is dependant normally up on fluid intake, environmental condition, diet and activity of the animal. The following data indicates the normal value of urine for different species

Dog ----- 12 – 30ml/lb body wt/24hrs

Cat ----- 4.5 – 9ml/lb body wt/24hrs

Horse ----- 2 – 8ml/lb body wt/24hrs

Swine ----- 2 – 14ml/lb body wt/24hrs

Cattle ----- 8 – 20ml/lb body wt/24hrs

Sheep ----- 4.5 – 14ml/lb body wt/24hrs

Value above or below the normal value can be considered as pathological but it should be combined with clinical and laboratory examination

Color-Can be observed in a test tube or in a urinometer tube. The following designations are used to observe the sample and correlated to the following terms.

✓Colorless	✓Greenish yellow	✓Blue
✓Pale yellow	✓Green	✓Milky
✓Yellow	✓Red	
✓Dark yellow	✓Reddish brown	
✓Yellow brown	✓Brown	

Interpretation

Yellow to amber – Normal

Colorless to pale yellow – dilute urine with low specific gravity and polyuria

Dark yellow or yellow brown – concentrated urine with a high specific gravity and small quantity

Yellow brown or greenish yellow – yellow green foam when urine is shaken

Urobilinoids – chromogen derived from heme green biliverdin yellow-brown-bilirubin-and urobilin

Cloudy – hematuria (clearer after centrifugation)

Translucent – hemoglobinuria

Brown to brownish black – hemoglobin up on standing bile large amounts, Normal horse urine is a yellowish color when voided, but it turns a deep brown color up on standing for a time due to oxidation of pyrocatechin

Green – bile biliverdin

Red to pink – phenothiazine

Transparency

Clear – freshly voided urine from normal animal is clear, except in the horse where it is normally thick and cloudy due to calcium carbonate (CaCO_3) crystals and mucus

Cloudy – not necessarily pathological as many samples may become cloudy

Epithelial cells – present in large numbers

Blood – red to brown color and smoky

Leukocytes – may produce milky, ropy appearance if large number

Bacteria – produce a uniform turbidity if in large number; the turbidity doesn't settle out and cannot be removed by filtration.

Mucus

Crystals

CaCO_3 – in fresh horse urine

Amorphous urate – white or pink cloud in acid urine

Amorphous phosphate – white cloud in alkaline urine

Specific gravity - determined by refractometer and indicator paper stripes. Normal value in most species will be in the range of 1.015 – 1.050 but values as high as 1.060 – 1.080 can occur.

Species	Range	Average
Horse	1.020 – 1.050	1.035
Cattle	1.025 – 1.045	1.035
Sheep and goat	1.025 – 1.045	1.030
Pig	1.010 – 1.030	1.015
Dog	1.015 – 1.045	1.025
Cat	1.020 – 1.040	1.030
Man	1.010 – 1.030	1.020

Table 7: Normal values of urine specific gravity in different species.

Average normal = 1.025

Odour

Normal odor – aromatic or acetone

Abnormal odor – aromatic odor of ketone bodies

Foam

Shake the sample and observe

- If the amount of foam produced is in excess and slow to disappear – proteinuria
- If the color of the foam great – yellow or brown – bile pigments
- If the color of the foam red to brown – hemoglobinuria

Microscopic Examination of Urine Sample

It is of great clinical importance and should never be omitted. Important structure to be include casts, erythrocytes, leukocytes, and bacteria

Method

- Agitate the urine to suspend any sediment that may settled to bottom
- Fill the centrifuge tube with urine and centrifuge for 3min at low rate of speed
- Pour all the urine and there is sufficient urine on the slide to drain to the bottom and suspend the sediment
- Pour a drop of sediment on a glass slide, and cover with a cover glass that has been wiped clean of oil and lint
- Examine under microscope with the lower power objective
- Finding should be reported as few, many or abundant
- If necessary stain with new methylene blue
- Organized sediments, epithelial cells

Chemical Examination of Urine

pH of urine

The normal hydrogen ion (pH) concentration, in the urine depends on the type of diet. Animals on vegetable diet produce alkaline urine while those on a high protein diet produce acidic urine.

Deep paper strip in to urine several times match the color of the paper strip with the chart 1min after wetting with urine.

Methods of measuring pH using pH meter

- Switch on the instrument and allow to “warm up” for a few minutes
- Rinse electrodes with distilled water and remove excess with soft paper, taking care not to touch the glass bulb
- Place electrodes into reference buffered solution
- Rinse as in “2” above
- Place in solution to be measured and take reading
- Rinse again and switch off instrument
- Keep electrodes wet, it should be not allowed to be dried

Protein Test

Methods of measuring pH using pH meter Robert’s test

Principle: Precipitation of protein by strong acid

Robert’s reagent: Conc. Nitric acid – 1 part

Saturated Magnesium sulphate – 5 part (770gm to 1 litre)

Procedure

- Place 2ml of Robert’s reagent in a test tube
- Layer 2ml of clear urine on the reagent by inclining the tube and allowing the urine to run slowly down the side from along dropped or pipette
- A positive test is indicated by a white ring at the zone of concentration, which should be read against a dark back ground and reported as

Negative - No ring at the zone of concentration

Trace - barely precipitate ring

+ Distinct narrow ring

++ Wider definite ring

+++ Very wide ring

++++ Thick, dense ring occupies most or all of urine layer

Note: In many clinical laboratories, Robert’s test is routine method as it is simple, quick and easy to read even when only a small amount of protein presents.

Glucose Test

Quantitative test of Benedicts

No glucose is present in the urine normally which passes glomerular filter, because it is completely absorbed in the tubules.

Method: Qualitative method

Principle: Reducing sugars present in the urine react with the copper sulphate to reduce the copric ions to cuprous oxide giving a color change depending on the amount of reducing substances present

Reagent used

Benedict’s reagent: Sodium sulphate -----173g

Sodium carbonate ----- 100g

Copper sulphate ----- 17.3g

Distilled water ----- 1000liter

Procedure

- Place 4ml of benedicts solution in a test tube
- Add 1ml of urine and mix very well
- Place the test tube in a boiling water for 5min and observe the color change

Result

Negative ----- blue

+ ----- trace light (light green)

++ -----	green (significant)
+++ -----	moderate orange to yellow
++++ -----	brick red (high concentration, serious)

Acetone (Ketone body)

Ross test

Principle: Sodium nitroprusside decomposed to sodium ferrocyanide, sodium nitrite and ferric hydroxide in an alkaline solution. These products are oxidizing agents that form a complex having a purple color in the presence of di-acetic acid and acetone

Reagent

It consists of a powdered mixture of: Sodium nitroprusside ----- 1 part
Ammonium sulphate ----- 100 parts

Procedure

- Place half of the powdered reagent in a dry test tube
- Add 5ml of urine to the tube and agitate
- Add 1ml to 2ml of conc ammonium hydroxide (NH₄OH) to form a layer above the mixture

In the field a flask of sodium hydroxide can substituted for the ammonium hydroxide solution and should be added before the tube is agitated

Result

Trace ----- very slight purple color
+ ----- slight purple color
++ ----- moderate purple color
+++ ----- dark purple color
++++ ----- dark purple color or black

N.B: This test is also effective when applied to milk. The ketone bodies include acetone acetoacetic acid (diacetic acid) and beta-hydroxybutric acid. A state in which these substances are present in increased amount in the blood and urine is called ketosis. Acetoacetic acid and beta-hydroxybutric acid from which acetone is derived is normal intermediate product of fat metabolism. When greater amounts of fatty acids are utilized with the production of more acetoacetic acid and beta-hydroxybutric acid can be oxidized by the tissues. These bodies accumulate in the blood and are excreted in the urine.

Rumen Function Test

Rumen fluid is collected by stomach tube; keep it at room temperature for 9 hours before examination.

Gross examination

Color: Color of the rumen content fluid depends up on the type of the feed. The color should be from green to live to brownish green. It will be green in grazing animals and grey in those animals that feed fodder betts, yellow brown in those that feed on silage or straw. Abnormally it may be greenish black in decomposition

Consistency: It is slightly viscous under normal condition. This viscosity is pronounced if large amount of saliva is included under the condition; it is advisable to discard the first part of the rumen fluid. It will be watery in case of acidosis and foamy in foamy bloat.

Odor:

- Under normal condition the odor of the rumen is aromatic odor and non replant
- Penetrating odor in acidosis
- Foul smelling in decomposition
- Different smell in active rumen

Total acidity of rumen contents

The pH of the rumen fluid varies from 5.5 – 7 which may be affected by saliva which contains bicarbonate. The PH of rumen fluid will be alkaline in active rumen during starvation and urea poisoning, the bacteria responsible for digestion and production of acid are weak and alkaline is predominates.

Urea poisoning, because of the breakdown of urea and formation of ammonia, the content of rumen becomes alkaline.

Indicator or dip stick test

Insert an indicator /deep stick in to the fluid and observe the color change

Titrate acidity test

- 0.1N NaOH

- Phenotaline solution (indicator)
- 10ml of rumen fluid

Procedure

- Pour 0.1N NaOH in the blurette, write down the volume of the solution
- Measure 10ml of rumen content in the beaker and add 1-2 drops of phenophtaline indicator and mix well
- Let 0.1N NaOH be in the beaker drop to neutralize and go on until fresh color fluid comes out

Result

Multiply total amount of NaOH by 10 i.e, $\text{NaOH /ml} \times 10$ clinical units under normal condition the clinical units range from 8 – units but in abnormal conditions it could be 10 – 70 units

Sedimentation Activity Time

Procedure

- Directly use rumen fluid or filter the fluid allow to stand for a few minutes
- The fine particles and infusoria settle down while floatation flows due to the production of gas by micro flora of rumen.

The time required for the setting down of infusoria and fine particles and floating of the production of gas by the microorganisms is known as the sedimentation activity time.

Under normal condition the SAT will be very weak floatation, slow or absent due to the absent of active microorganism, example in starvation.

In acidosis Lactobaccili are very active and there is large amount of gas production and floatation are very quick and large.

Bacteria Examination

Purpose: To determine the dominate bacteria in the ruminal fluid

Method: Gram stain

Materials: Gram staining dyes (GV, iodine, safranin, methanol or acetone), slide, microscope

Procedure

- Take a rumen fluid on the slide and make a smear
- Dry and stain with gram stain
- Observe under oil immersion for the presence or absence of the microorganisms. The bacteria in the rumen is referred as leading bacteria
- Observe the gram negative and gram positive bacteria, write the dominate and the ratio
- Under normal condition gram negative bacteria are the dominate
- In the rumen of the animals feed on roughage, the large streptococci kidney shaped cocci, saracia are predominantly present
- Under acidosis condition Gram positive bacteria predominantly present

Microscopic Examination of Rumen Content Protozoan's

There are two form of protozoa to be predominate in rumen fluid

- Ciliates
- Flagellates

The ciliates are important because massive in numbers than the flagellates. There are 10 – 20 millions species which inhibits in the rumen as far as the physiological significance of these protozoan are considered to be not use full. However they are important in stabilizing bacterial digestive process.

Procedure

- Take one or two drops of rumen fluid on the slide
- Cover with cover slip and warm around 13°C
- Observe under microscope
- Grade the number of protozoa

Result

- If it is abundant----- + + +
- If it is moderate ----- + +

- If it is few ----- +
- If no protozoa ----- -ve

Interpretation

The size of the protozoa could be large or small and medium. The large protozoa are the first to die. There is no digestion in the rumen in the death of protozoa. Therefore, observe the proportion of death to alive one, in case of acidosis, No digestion i.e., no protozoa

Cellulose Digestion Test

Procedure

- Take 10ml of rumen content in the test tube
- Add about 0.3ml of 10% glucose solution
- Suspend cotton thread to the solution and incubate in 37°C for 48 hrs
- The expected result is the action of microflora on the thread

Result

If the rumen is active, there will be break down of the thread

Pancreatic Function Test

Microscopic examination

Fat test

Purpose: To determine the presence of fat substance in the fecal sample

Reagent preparation: Sudan III or Sudan IV stain. Take equal parts of 70% alcohol and acetone with excess of Sudan III or Sudan IV stain.

Sample required: Feces

Procedure

- Mix a small portion of feces with water or saline on the slide
- Combine the feces with equal parts of Sudan III or IV stain
- Examine under the low power objective ($\times 10$) after placing a cover glass on the mixture

Result

Neutral fat will appear as orange as red globules, which vary in size and shape and indicate deficiency of pancreatic lipase, i.e. undigested fat in feces indicates absence of pancreatic lipase which results pancreatic insufficiency

Trypsin

Film test

Test for proteolytic activity employing gelatin digestion (for small animals)

Procedure

- Bring 9ml of 5% sodium bicarbonate to 19ml total volume by adding feces and mix well
- Place $\frac{1}{2}$ to $\frac{1}{4}$ of the mixture in a vial or test tube, place a narrow strip of radiographic film in the vial
- Incubate at 37°C for half an hour
- Wash the film under a gentle stream of tap water

Result

A cleared area on the submerged portion of the film indicates the presence of Trypsin.

With a deficiency of trypsin the emulsion will only be water marked or partially removed

Gelatin Test Method

This test is more sensitive than the film test method

Preparation

Mix 7.5gm of gelatin powder with distilled water in a 100ml of volumetric flask, bring 199ml, boil it, and distribute 2ml in a test tube and keep it in the refrigerator

Procedure

- Warm two test tube each containing 2ml of 7.5% gelatin in 37°C to liquefy

- Bring 9ml of water to a total volume of 100ml by adding feces and mix well
- Add 2ml of the feces mixture as described above to one gelatin tube
- Add 2ml of 5% sodium bicarbonate (NaHCO₃) to the other gelatin tube for the control, mix well
- Incubate both tubes at 37°C for 1hr
- Refrigerate or place in cold water (4°C) for 20 minutes

Result

- Failure to gel formation indicates the presence of trypsin, which has digested the gelatin i.e No pancreatic insufficiency
- Gel formation (coagulation) indicates the absence of trypsin. i.e pancreatic insufficiency if tests are repeatedly deficient.

References

1. Benjamin MM (1978) Outline of Veterinary Clinical Pathology. (3rd Edn), Ames, IA, the Iowa State University Press.
2. Denny Meyer, John W Harvey, Ray Kersey (1997) Veterinary Laboratory Medicine: interpretation and Diagnosis, Second Edition: Elsevier Health Sciences.
3. Jiro Jerry Kaneko, John W Harvey, Michael I Bruss (2008) Clinical Biochemistry of Domestic Animals, Sixth Edition.
4. Kenneth S Latimer (2011) Duncan and Prasse's Veterinary Laboratory Medicine: Clinical Pathology, Fifth Edition.
5. Krehl Rudolf von (1907) The principles of clinical pathology, a text-book for students and physicians; Philadelphia Lippincott.
6. Marion L Jackson (2007) Veterinary Clinical Pathology: An Introduction, First Edition.
7. Marry Anna Thrall (2006) Clinical Case Presentations for Veterinary Hematology and Clinical Chemistry.
8. Marry Anna Thrall (2006) Veterinary Hematology and Clinical Chemistry.
9. Robert J Duncan, Edward A Mahaffey, Keith W Prasse (1994) Veterinary Laboratory Medicine: Clinical Pathology, Third Edition.
10. Steven E Crow, Sally O Walshaw, Jennifer E Boyle (2009) Veterinary Clinical Pathology. Manual of Clinical Procedures in Dogs, Cats, Rabbits, and Rodents.
11. Steven L stockham, Michael A Scott (2002) Fundamentals of Veterinary Clinical Pathology, First edition.
12. Steven L Stockham, Michael A Scott (2008) Fundamentals of Veterinary Clinical Pathology Second Edition.

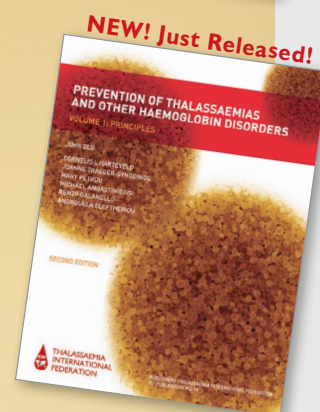
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