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International Journal of Veterinary Science and Research

DOI: http://dx.doi.org/10.17352/ijvsr

Special Issue: Manual guidance of veterinary clinical practice and laboratory

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Received: 14 May, 2018
Accepted: 13 August, 2018
Published: 14 August, 2018

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Research Article

Veterinary laboratory guidance

The Veterinary Diagnostic Laboratory has the great role in diagnosing medical testing for infectious agents, toxins, and other causes of disease in animal diagnostic samples which submitted by veterinary practitioners serving animal owners, public health officials, wildlife management, and scientists with research projects. This Veterinary Diagnostic Laboratory guidance focused on the discipline of special veterinary faculty like pathology, clinical pathology, microbiology, virology, immunology, parasitology, and serology.

Principle of biosafety in laboratory

The principle gives some awareness for the lab worker and student, how to operate in laboratory and what is the prohibition act in laboratory and etc. The main safety in laboratory is listed as following [1-3]:

- ✓ Personnel must wash their hands after handling infectious materials and animals, and before they leave the laboratory working areas.
- ✓ Safety glasses, face shields (visors) or other protective devices must be worn when is necessary to protect the eyes and face from splashes, impacting objects and sources of artificial ultraviolet radiation.
- ✓ It is prohibited to wear protective laboratory clothing outside the laboratory, e.g. in canteens, coffee rooms, offices, libraries, staff rooms and toilets.
- ✓ Open-toed footwear must not be worn in laboratories.
- ✓ Food (including chewing gum, candy, throat lozengesand cough drops) and/or drink shall not be stored or consumed in laboratories.
- ✓ Smoking and/or application of cosmetics shall not take place in the laboratory; Pipetting shall not be done by mouth.
- ✓ Eating, drinking, smoking, applying cosmetics and handling contact lenses is prohibited in the laboratory working areas.
- ✓ Appropriate gloves must be worn for all procedures that may involve direct or accidental contact with blood, body fluids and other potentially infectious materials or infected animals. After use, gloves should be removed aseptically and hands m ust then be washed.
- ✓ Storing human foods or drinks anywhere in the laboratory working areas is prohibited.
- ✓ Protective laboratory clothing that has been used in the laboratory must not be stored in the same lockers or cupboards as street clothing.
- ✓ The laboratory should be kept neat, clean and free of materials that are not pertinent to the work.
- ✓ Work surfaces must be decontaminated after any spill of potentially dangerous material and at the end of the working day.
- ✓ All contaminated materials, specimens and cultures must be decontaminated before disposal or cleaning for reuse.
- ✓ The laboratory should be easy to clean, with surf aces that are impervious to water and resistant to chemicals. There shall be a wash-hand basin and emergency shower, including an eye bath, in each laboratory suite as appropriate for the chemicals and other hazards present. Procedures shall be established for frequent cleaning and disinfection during and at the end of the work period;
- ✓ Personal protective equipment such as long-sleeved lab coats or gowns, closed-toe footwear, disposable gloves, masks, safety glasses, face shields, and oro-nasal respirators, as appropriate, shall be worn in the laboratory and removed when leaving the laboratory.
- ✓ The laboratory door should be closed when work is in progress and ventilation should be provided by

Citation: Tagesu A (2018) Veterinary laboratory guidance. Int J Vet Sci Res s1: 031-044. DOI: http://dx.doi.org/10.17352/ijvsr.s1.105

extracting air from the room (Where biosafety cabinets are used, care shall be taken to balance ventilation systems.

- ✓ Emergency response plans should be developed to deal with the biohazard of spills. Some of the items addressed in the plans should include having effective disinfectant available for cleaning spills, removal of and decontamination of contaminated protective clothing, washing of hands, and cleaning and disinfection of bench tops.
- ✓ Used laboratory glassware and other contaminated material shall be stored safely. Materials for disposal shall be transported without spillage in strong containers. Waste material should be autoclaved, incinerated or otherwise decontaminated before disposal. Re usable material shall be decontaminated by appropriate means.
- ✓ No infectious material shall be discarded down laboratory sinks or any other drain.
- ✓ Any accidents or incidents shall be recorded and reported to the Safety Officer.
- ✓ Some common terms used in veterinary clinical diagnostic and laboratory practice are listed below [1,4]:

- Diagnosis is an art of precisely knowing the cause of a particular disease. (Dia =thorough; gnosis = knowledge). The diagnosis is based on accurate history, careful examination of animal, collection of material for laboratory examination and correlation and interpretation of findings.
- *Snap diagnosis:* This is to give an opinion about the cause of disease by merely looking at the animal. It is often erroneous unless the animal is showing pathognomonic symptoms.
- *Tentative diagnosis:* The tentative diagnosis is based on clinical symptoms and physical examination. It is to give an approximate cause of disease.
- *Symptomatic diagnosis:* The symptomatic diagnosis is based on a few important symptoms without knowing the cause of disease.
- *Confirmatory diagnosis:* It is based on clinical, physical and laboratory findings. It includes the exact cause of disease and determination of which one of several diseases may be producing the symptoms.
- *Differential diagnosis:* This is the process of exclusion for differentiating among diseases having similar symptoms or closely related diseases, e.g. diabetes mellitus and diabetes insipidus.
- *Test therapy diagnosis:* The diagnosis of disease depends on the response of the animal to a particular drug or medicine.
- *Clinical diagnosis:* This diagnosis is based on the inspection of animal by looking the clinical symptoms.
- *Physical diagnosis:* Tlus diagnosis is based on examination of animal by physical methods like palpation, percussion and auscultation.
- *Laboratory diagnosis:* The laboratory diagnosis is based on laboratory findings like examination of clinical samples such as serum, feces, blood etc.
- *Symptoms:* Any subjective evidence of disease of an animal characterized by a change in patient's condition indicative of some bodily or mental state as told by the owner.
- *Signs:* An indication of the existence of something, any objective evidence of d~seasew, luchis perceptible to the veterinarian. These are based on the veterinarians' observations.
- General symptoms: The general symptoms include symptoms involving whole body like fever/rise in temperature.
- *Systemic symptoms:* These symptoms are an indication of involvement of a system of body like vomiting, diarrhoea indicating the involvement of digestive system; respiratory symptoms, nasal discharge indicate the affections of lungs and respiratory passage.
- *Pathognomonics symptoms:* These symptoms are specific for a particular disease and based on that one can diagnose the disease with confirmation, e.g. rusty brown nasal discharge in equine infectious anemia.
- *Lesions:* Lesion is the pathological alterations in structure or function and can be detectable either by naked eyes or microscopically.

The veterinary diagnostic laboratory guidance is the best guidance of the clinician and veterinarian who work over research and laboratory. Therefore, the most common examination undertaken during practice is as listed below:

- ✓ Examination of blood
- ✓ Examination of feces
- ✓ Examination of urine
- ✓ Examination of cerebrospinal fluid
- ✓ Examination milk
- ✓ Examination skin scraping
- ✓ Antimicrobial sensitivity test
- ✓ Serological and immunological
- ✓ Molecular biology examination
- ✓ Postmortem/necropsy examination

Citation: Tagesu A (2018) Veterinary laboratory guidance. Int J Vet Sci Res s1: 031-044. DOI: http://dx.doi.org/10.17352/ijvsr.s1.105

Examination of Blood in Domestic Animals

General scientific fact about blood

Blood is a bodily fluid in animals that delivers necessary substances such as; nutrients and oxygen to the cells and transports metabolic waste products away from those same cells. Blood which is a vital special circulatory tissue is composed of cells suspended in a fluid intercellular substance (plasma) with the major function of maintaining homeostasis [5]. The components of blood include plasma (the liquid portion, which contains water, proteins, salts, lipids, and glucose), red blood cells and white blood cells, and cell fragments called platelets [6].

Blood plays an important role in regulating body systems and maintaining homeostasis. It performs many functions within the body including:

- Supplying oxygen to tissues (bound to hemoglobin, which is carried in red cells)
- Supplying nutrients such as glucose, amino acids, and fatty acids either dissolved in the blood or bound to plasma proteins (e.g., blood lipids)
- Removing waste such as carbon dioxide, urea, and lactic acid
- Immunological functions, including circulation of white blood cells and detection of foreign material by antibodies
- Coagulation, which is one part of the body's self-repair mechanism (blood clotting by the platelets after an open wound in order to stop bleeding)
- Messenger functions, including the transport of hormones and the signaling of tissue damage
- Regulating body pH
- Regulating core body temperature
- Hydraulic functions, including the regulation of the colloidal osmotic pressure of blood.

Medical terms related to blood often begin with hemo- or hemato- (also spelled haemo- and haemato-), which is coined from the Greek word α (haima) for "blood". In terms of anatomy and histology, blood is considered a specialized form of connective tissue, given its origin in the bones. Blood cells produced at different stages of development differ in morphology and function. Thus, primitive (fetal) cells fabricated early in gestation have markedly different properties from adult counterparts produced during late gestation and in postnatal life (Table 2). Primitive erythrocytes (RBCs) are formed in the yolk sac, whereas definitive RBCs are produced by the liver and later spleen and bone marrow. Primitive RBCs are nucleated in circulation until approximately day 12.5 (E12.5) of gestation, after which nuclei gradually become condensed before being shed between E14.5 to E16. 5 [7].

Both primitive and definitive RBCs have basophilic cytoplasm when first produced due to abundant rough endoplasmic reticulum [8]. Haematogenesis is the process of differentiation of blood from bone marrow; it is regulated by erythropoietin (EPO) hormone secrated from kidney. Secreted molecules also are important regulators of hematopoietic development during gestation. Erythropoietin (EPO) sustains both primitive and defi nitive erythropoiesis by stimulating proliferation and differentiation of immature primitive and defi nitive RBCs [9]. Different cell lineages occupy specific locations: granulocytes, lymphocytes, and macrophages are concentrated near the endosteum and arterioles, and megakaryocytes and erythroid cells are located near venous sinuses [10]. Hematopoietic cells are derived from a common pluripotent stem cell which gives rise to lymphoid and myeloid progenitor cells. Lymphoid progenitor cells generate lymphocyte progeny, whereas the myeloid progenitor cells generate erythroid cells, megakaryocytes, basophils, eosinophils, and a common granulocyte macrophage cell that produces neutrophils and macrophages (Figure 3) [11].

Stages of erythropoiesis include rubriblasts, prorubricytes, rubricytes, metarubricytes, reticulocytes, and mature RBCs [12]. As erythroid precursors mature, the cells become smaller, the nuclear to cytoplasmic ratio decreases, the cytoplasm becomes less basophilic and more polychromatophilic, and the nuclear chromatin becomes condensed. In mammals, the nucleus is extruded before maturation to a mature RBC [12]. Red blood cells, or erythrocytes (erythro- = "red"; -cyte = "cell"), specialized cells that circulate through the body delivering oxygen to other cells, are formed from stem cells in the bone marrow. In mammals, red blood cells are small, biconcave cells that, at maturity, do not contain a nucleus or mitochondria; they are only 7–8 μm in size. In birds and non-avian reptiles, red blood cells contain a nucleus.

The red coloring of blood comes from the iron-containing protein hemoglobin.The principal job of this protein is to carry oxygen, but it transports carbon dioxide as well. Hemoglobin is packed into red blood cells at a rate of about 250 million molecules of hemoglobin per cell. Each hemoglobin molecule binds four oxygen molecules so that each red blood cell carries one billion molecules of oxygen. There are approximately 25 trillion red blood cells in the five liters of blood in the human body and also for domestic animals is detailed in table 1, which could carry up to 25 sextillion (25 × 1021) molecules of oxygen at any time. In mammals, the lack of organelles in erythrocytes leaves more room for the hemoglobin molecules. The lack of mitochondria also prevents use of the oxygen for metabolic respiration. Only mammals have anucleated red blood cells; however, some mammals (camels, for instance) have nucleated red blood cells [13].

The advantage of nucleated red blood cells is that these cells can undergo mitosis. Anucleated red blood cells metabolize anaerobically (without oxygen), making use of a primitive metabolic pathway to produce ATP and increase the efficiency of oxygen transport. Not all organisms use hemoglobin as the method of oxygen transport. Invertebrates that utilize

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hemolymph rather than blood use different pigments containing copper or iron to bind to the oxygen. Hemocyanin, a blue-green, copper-containing protein is found in mollusks, crustaceans, and some of the arthropods. Chlorocruorin, a green-colored, iron-containing pigment, is found in four families of polychaete tubeworms. Hemerythrin, a red, iron-containing protein, is found in some polychaete worms and annelids [13].

Leukocytesor white blood cells (from the Greek word *leuco*, white) differ considerably from erythrocytes in that they are nucleated and are capable of independent movement to exit blood vessels. Leukocytes may be classified as either granulocytes or agranulocytes based on the presence or absence of cytoplasmic granules that stain with common blood stains, such as Wright's stain. These stains contain an acid dye, eosin, which is red, and a basic dye, methylene blue, which is bluish [14].

Granulocytes are named according to the color of the stained granules (i.e., neutrophils, which have granules that stain indifferently; eosinophils; and basophils). The differentiations of white blood cell in domestic animals are detailed in figure 2. The nuclei of granulocytes appear in many shapes and forms, leading to the name polymorphonuclear leukocytes (from the Greek poly, many; morpho, form). However, the term is commonly used to indicate neutrophils, because they are normally the most prevalent granulocyte. Monocytes and lymphocytes are the two types of agranulocytes (Figure **1**) [14].

Blood platelets, also called thrombocytes, are fragments of megakaryocytes, large cells formed and residing in the bone marrow. Thrombocytes are the smallest of the formed elements in the blood. They are surrounded by a plasma membrane and contain some organelles, but not nuclei. The appearance of platelets in a stained smear may be considerably different from

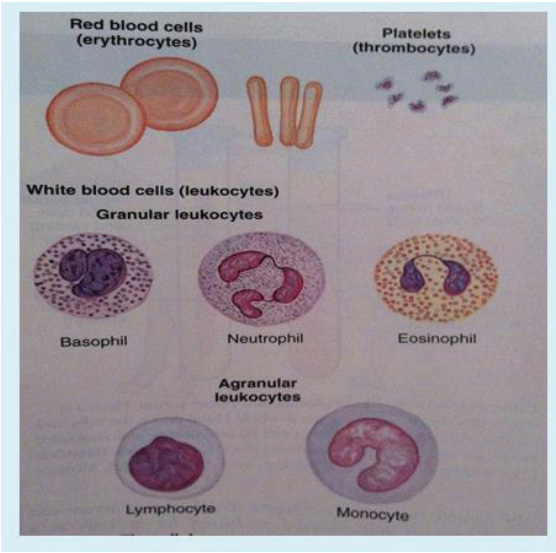

Figure 1: Normal blood cells (white, red and platelet) of animals.

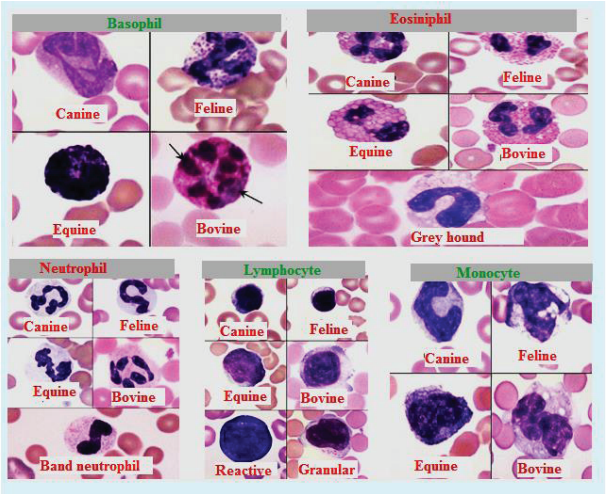

Figure 2: Leukocytes of domestic animals with their comparison [15].

Table 1: The numbers per microliter of blood cell and platelets in domestic animal [14].

Blood elements	Horse	Cow	Dog	Chicken
Erythrocytes	8-11x 10^6	6-8 x10^6	6-8 x10^6	2.5-3 x10^6
Total leukocytes	8-11 x10^3	7-10 x10^3	9-12 x10^3	20-30x10^3
Neutrophils	4-7 x10^3	2-3.5 x10^3	6-8.5 x10^3	5-10 x10^3
Lymphocytes	2.5-4 x10^3	4.5-6.5 x10^3	2-3.5 x10^3	11-18 x10^3
Eosinophils	200-500	150-500	200-500	600-2,000
Monocytes	400-500	350-500	450-600	2-3 x10^3
Basophils	<100	<100	<100	200-900
Platelets	150-450 x10^3	300-500 x10^3	300-500x10^3	25-40 x10^3

Table 2: The site of hematopoiesis/generation of RBC in domestic animals.

Age of animals	Site of hematopoiesis
Embryo	Yock sack then liver
3rd to 7th month*	Spleen
4th and 5th months*	Marrow cavity (granulocytes and platelets.)
7th months*	Marrow cavity (erythrocytes.)
Birth	Mostly bone marrow; spleen and liver when needed.
Birth to maturity	Numbers of active sites in bone marrow decreases but retain ability for hematopoiesis.
Adult	Bone marrow of skull, ribs, sternum, vertebral column, pelvis, proximal ends of femurs.

their actual appearance in circulating blood, where they are oval disks. In smears they may appear as circular disks, star-shaped fragments, or clumps of irregular shape. Substances released by platelets and lodged on their surface membranes stimulate clotting and help cause local constriction of the injured blood vessel [15].

Blood cell collection

Equipments and reagents required for hematological blood examination.

- Hematocrit centrifuge
- Compound microscope
- Sahlis instrument
- Capillary tube
- Hematocrit reader
- Distilled water

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- 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(Figure 4)
- Vacutainer needles
- syringes
- needle holder
- Gloves
- Blood samples
- Staining racks and others

The blood is collected from animals through puncture of vein using syringe and needle. In laboratory animals or poultry, however, it is collected directly from the heart [17-24]. The sites of blood collection in different animal species are described in figure 5 and table 3.

Anatomical Location of the Veins of Domestic Animals

Jugular vein

The jugular vein is situated in the jugular groove, over the trachea and carotid artery on both sides of the neck [25]. Before collection of blood, the hairs of the area are removed with the help of scissors and the area is shaved with razer. The antiseptics like spirit is applied over the area of vein puncture and the animal is properly restrained. The vein is raised by applying pressure at ventral point of puncture. The vein can

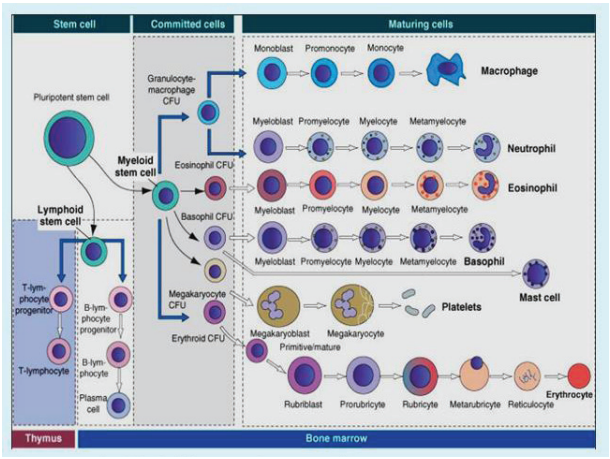

Figure 3: Mode of blood cell formation or haemopoesis [16].

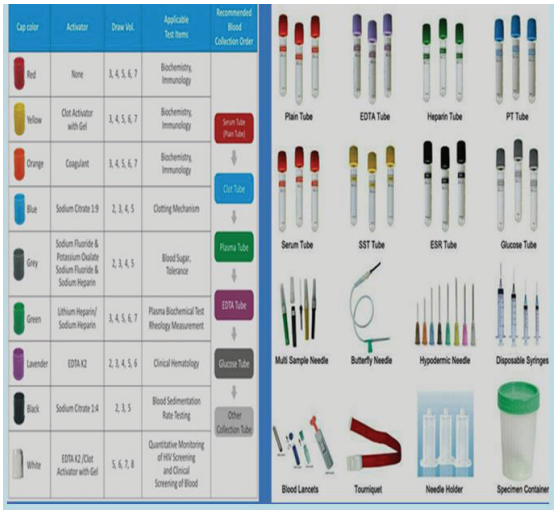

Figure 4: Equipment of blood collection in domestic animals.

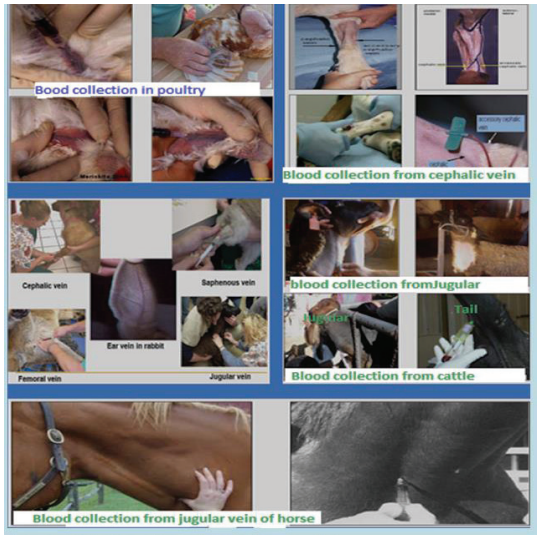

Figure 5: Anatomical site of blood collection in domestic animals.

Table 3: Common site of blood collection in domestic animals.

Animals species	Site of blood collection
Cattle	Jugular vein, ear vein
Horse	Jugular vein
Camel	Jugular vein
Sheep	Jugular vein
Goat	Jugular vein
Pig	Ear vein, anterior venacava
Dog and Cat	Cephalic vein, Recurrent tarsal vein
Poultry	Wing vein, Heart, Small bird from: Brachial, jugular, and femoral veins.

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be felt by finger tapping. Blood is collected by using a 16 gauge needle in large animals and 18 gauge needles in small animals. The needle is inserted in the vein by force in an angle; blood can be collected directly in the collection tube or by applying syringe over needle to suck the blood. After collection of blood, the pressure is released and needle is removed. Apply antiseptics at the site of vein puncture. In casted animals, the neck should be turned upwards as it helps in distention of vein for puncture [26]. The right jugular is the largest and by far the most preferred site for avian blood collection. It is easily visualized by applying a small amount of alcohol to the featherless tract on the right side of the neck. The vein should be occluded with a thumb or forefinger at the level of the thoracic inlet prior to venipuncture [27].

Ear vein

The ear vein technique is a simple, quick and reliable method of taking blood samples from cattle for determination of the haematocrit values of blood [28]. Small amounts of blood (1-2 ml) can be collected from ear vein [29,30]. The area should be cleaned and shaved properly at the dorsal side of the ear. Antiseptics should be applied over the area. The vein is raised by applymgpressure of thumb and index finger of left hand and blood is collected using a 20 or 22-gauge needle with syringe. After collection of blood, the pressure is released and the vein puncture site is rubbed with cotton dipped in spirit. Ear blood was more sensitive than jugular blood for the detection of parasitacmia [31].

Cephalic vein

The cephalic vein is situated in the anterior surface of the forelegs, from where hairs should be removed and the area is shaved [26]. For proper disinfection, 70% alcohol spirit is applied over the area. Use a torniquet around the elbow to raise the vein. Vein is felt by fingertip and a needle of 20-22 gauge is inserted along with synnge. The needle should be in the right side of the vein and a click like sound appears when the needle penetrates the vein. Collect blood in desired quantity and remove the torniquet, needle and syringe. Apply antiseptic at the site of puncture.

Recurrent tarsal vein

This vein is situated on the lateral side of the hock joint where it takes a round [26]. The pressure should be applied over the shfle joint. The area must be cleaned, shaved and properly disinfected. The blood is collected with a 20 gauge needle with syringe. After collection of blood, pressure is released and needle is removed. The antiseptics are applied over the vein puncture area.

Poultry

In poultry, small amount of blood (1-2 ml) can be collected from wing vein [32]. However, if more blood is required, it should be collected directly from the heart. The wings are extended and the area should be cleaned. Feather or fluffs are removed from the site and then the vein is raised by applying pressure at the root of wing. The needle (20 gauges) is inserted in the vein and blood is collected slowly. Remove the pressure, before withdrawal of needle and apply proper disinfectant vein puncture site. For the heart puncture, the bird is placed on its back with extended neck and wings against the chest [33].

The needle (20 gauges) along with syringe is applied from dorsal side in the groove of neck and the needle is directed forward in slightly raised position. When it punctures the heart, blood comes out in the syringe. Collect the desired quantity of blood and remove the needle. Apply proper disnfectant on the area of needle puncture. The other method for collection of blood from poultry is from jugular vein. For Blood collection from jugular vein of birds, first the neck is cleaned and fluffs are removed and the area is properly disinfected.

The head of the bird is held at its base between fore and middle fingers and then with the help of the thumb the jugular vein is so placed that it rests on the ridge or the solid background of cervical vertebrae. The needles are inserted into the vein and collect the blood in syringe. After collection of blood, remove the needle and syringe and apply disinfectant at the site of vein puncture. After the collection of blood from animal, the needle is removed from syringe and the blood is slowly transferred to the collection tube in order to avoid the breakage of erythrocytes and formation of hemolysis 23.

Interpretation of Examined Blood

The wet film of blood is examined for the demonstration of movements of microfilaria or hypanosomes. For this a drop of fresh or anticoagulant added blood is placed over a clean and dry slide. It is covered with a cover slip and examined under microscope. The parasites are seen moving in between the erythrocytes. The trypanosomes are recognized by their swirling movements. These parasites can also be demonstrated by lysis of blood using saponin.

Interpretation of red blood cell and white blood cell

Increased total erythrocyte count (erythrocytosis) is reported in following conditions. Dehydration, hemoconcentration, exercise, occlusion of vein for a longer period, brucellosis, campylobacteriosis, leptospirosis, rinderpest, pasteurellosis, acute poisoning, chronic heart disease, pulmonary fibrosis, shock, vomiting, diarrhoea, hypoxia [34-37].

Decreased total erythrocytic count (erythropenia) occurs in following conditions: Anemia, anaplasmosis, babesiosis, leptospirosis, copper, lead and phenothiazine poisoning, equine infectious anemia, defective blood formation, leukemia, hemorrhage, aflatoxicosis, coccidiosis, fasciolosi [38,39].

The total leucocyte count is performed by hemocytometer using a WBC diluting pipette. The blood is sucked in pipette up to 0.5 mark and then WBC diluting fluid, as in case of total erythrocyte count, upto 11 marks. Remove the rubber tubing, keeping the pipette in horizontal position. Mix the contents of pipette by rotating the pipette between the palms and discard the first few drops. Fill the space in between coverslip and counting chamber and wait for 1-2 min for settling of cells. Put the counting chamber under microscope and count

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the cells in four large corner squares of the ruled area under low power. Calculate the total leucocytes per cubic mm of blood by multiplying 50 with the count. The white blood cell (WBC, leukocyte) count of birds is obtained using manual techniques because the presence of nucleated erythrocytes and thrombocytes interfere with the counting of white blood cells using electronic cell counters [40,41].

For total leucocyte count in poultry, the diluting pipette of erythrocytes is used because of high leucocytic count. Like total erythrocytic count, fill the RBC diluting pipette uptomark 0.5 and fill it with "WBC diluting fluid-poultry" upto mark 101. Remove the rubber tubing and mix the contents, discard a few drops of contents and fill the counting chamberas described earlier. After 1-2 min, count the cells under low power/high power in four corner primary squares of the counting chamber and calculate the number of cells by multiplying 5000 to get total leucocyte count per cumm of blood [40].

Interpretation of white blood cell

Leucopenia is the decrease in total leucocyte count and is observed in following conditions: Canine distemper, infectious canine hepatitis, feline panleukopenia, mucosal disease, hog cholera, swine influenza, chronic intoxication of lead, bismuth, arsenic, mercury, pesticides, prolonged antibiotic therapy, rinderpest, bovine malignant catarrh, extreme debility and loss of resistance, expo sure of X-rays, salmonellosis, toxoplasmosis, PPR, brucellosis, chlamydiosis, leishmaniosis, tuberculosis, blue tongue, lymphosarcoma, hepatic cirrhosis, mastitis, copper deficiency, afla toxicosis, BHV-1 infection, bracken fern poisoning [37,42].

Leucocytosis is the increase in number of leucocytes and is observed in following disease conditions. Pasterellosis, pto-spirosis, salmonellosis, local infection of *streptococci, staphylo-cocci,* and *coynebacteriurn,* uremia, diabetes, gout, malignancy, viral infections, rabies, histoplasmosis, tuberculosis, strangles, blood protozoan infections, colibacillosis, chronic enteritis, bovine, immunodeficiency syndrome, acidosis, burn, gangrene [37,42,43].

Following are some factors which affect the total leucocytic counts:

- *Age:* TLC high at birth in pups, calves TLC low at birth in piglets
- *Species:* Bovines have more lymphocytic count wl-ule canines have more neutrophilic count.
- *Excitement:* Excitement causes an increase in TLC.
- *Pregnancy:* During pregnancy total leucocyte count increases.
- *Estrus:* During estrus TLC increases.
- *Digestion:* In dogs and pigs after eating food, there is increase in total leucocyte count and neutrophilic count.

Differential leucocyte count and interpretation

For differential leucocyte count, a thin blood smear is prepared on glass slide. A drop of fresh or anticoagulant mixed blood is placed on one corner/end of slide and spread as smear with the help of another slide using its thin edge at an angle of 450. Smear should be uniform and thin and it should not have any air bubble. Dry the smear in air and mark the identification number in the thck portion of the smear. Thereafter fix the smear in methanol for 5 min and dryin air. Stain the smear with Giemsa stain diluted to 1:10 in distilled water for 30 min. Wash the slides, dry in air and examine under oil immersion of the microscope. Count at least 200 cells by battlement/ zigzag method. Cells counted are neutrophils, lymphocytes, eosinopluls, monocytes and basophils.

The interpretation is as the following listed [44-47].

Lymphocytosis: Increased number of lymphocytes is observed in viral infections, tuberculosis, brucellosis, hypothyroidism, following vaccination, Leukemia, Adrenocortico insufficiency etc.

Lymphopenia: Decrease number of lymphocytes is observed in canine distemper, infectious canine hepatitis, corticosteroid therapy, hypothyroidism, BHV-1 infection, Q-fever, foot and mouth disease, mucosal disease etc.

Neutrophilia: Increased number of neutrophils is observed in septicemic diseases, uremia, gout, coronary thrombosis, pyogenic infections, acute inflammation, cancer, arthritis, pyotnetra, post-surgical operation, coronary thrombosis, pregnancy, calf diphther rhehumatic fever.

Neutrophilia (Shift to left): Increased number of neutrophils with immature cells is observed in leptospirosis, traumatic reticulopericarditis (TRP), metritis, canine distemper, glanders, endocardi'tis, synovitis etc.

Neutropenia: Decrease number of neutrophils is observed in pasteurellosis, bovine viral diarrhoea, and infectious canine hepatitisetc.

Eosinophilia: Increase number of eosinophils is observed in allergy, parasitic infection, skin disease, anaphylactic reaction, convalescence, eosinophilic myositis and granulocytic eosinophlic leukemia.

Basophilia: Inverse innumber of basophls is observed in Pox infections, sinus~tiss, pleenectomy, cirrhosis, Hodgkin's disease, introduction of foreign protein.

Monocytosis: Increase in number of monocytes is observed in tuberculosis, brucellosis, trypanosomiasis, convalescence, monocytic leukemia, carbonate and chloride poisoning, Sys-temic fungal diseases (histoplasmosis, blastomycosis, crypto-coccosis, coccidioidomycosis, aspergillosis),

Hyperhemoglobinemia: Increase in hemoglobin is observed in polycy themia, dehydration, leptospirosis, equine influenza, acid indigestion.

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Hypohemoglobinemia: Decreased in hemoglobin content is observed in anemia, theileriosis, strangles, anaplasmosis, Degnala disease, fasciolosis.

Erythrocytc sedimentation rate

The erythrocyte sedimentation rate (ESR) is measured by using Westergren pipette [48,49]. Suck the anticoagulant mixed blood in Westergren pipette upto mark '0' and fix it in stand in vertical position. Leave this for one hour at room temperature. After one hr record the reading on graduated pipette, it is the mm fall of erythrocytes per hour. Care should be taken that there should not be any air bubble in the pipette. Pipette should be filled exactly to '0' mark. ESR should be performed with one hour of blood collection.

The values of ESR are increased intuberculosis, carcinoma, nephritis, gout, heavy metal toxicity, rheumatoid arthritis, pleurisy, canine distemper, pyometra, pencarditis, peritonitis, leptospirosis, filariasis, pneumonia, fracture, trypanosomiasis, equine infectious anemia and parasitic diseases. Decreased erythrocyte sedimentation rate has been recorded in hemolytic jaundice, sickle cell anemia and in brucellosis [49-51].

Increased hematocrit value: Dehydration (Increased total protein and/or albumin, Increased sodium and chloride ,Increased BUN and creatinine, increased urine specific gravity) , ketosis, leptospirosis, rotavirus infection, influenza, rinderpest, Renal: cysts, tumors, hydronephrosis, Erythropoietin-secreting tumors (paraneoplastic), High altitude, Hyperthyroidism, Chronic lung disease (Increased reticulocyte count and nucleated red blood cells, Decreased PO_2 on arterial blood gas, low SpO_2 on pulse oximeter, Pulmonary pathology on thoracic radiographs), Chronic cardiac disease [36,52,53].

Decreased hematocrit value: Anemia, parasitic infection, muscular dystrophy, gestation, lactation, theileriosis, strangles, anaplasmosis, blue tongue, endocarditis, hemolysis(Immune-mediated (IMHA), Infectious: hemotropic mycoplasma, rickettsial diseases, babesiosis, cytauxzoonosis, heartworm, Zinc toxicity) and Bone marrow disease/myelophthisis (e.g. lymphoproliferative, myeloproliferative disorders, metastatic neoplasia, myelofibrosis) 35,53.

Blood smear

Principle of smear preparation

A small drop of blood is placed near the frosted end of a clean glass slide. A second slide is used as a spreader. The blood is streaked in a thin film over the slide. The slide is allowed to air-dry and is then stained. EDTA anticoagulated blood is preferred. Blood smears can also be made from fingerstick blood directly onto a slide. Three methods may be used to make blood smears [54, 55]:

- The cover glass smear,
- The wedge smear and
- The spun smear. The spun smear requires an automatic slide spinner. For the purpose of lab exercise, we will use the wedge smear (Figure 6).

Procedure how to make blood smear [56-59] (Figure 7).

- Using the stopper piercer, place a drop of blood, about 2 mm in diameter approximately inch from the frosted area of the slide.
- Place the slide on a flat surface, and hold the narrow side of the nonfrosted edge between your left thumb and forefinger.
- With your right hand, place the smooth clean edge of a second (spreader) slide on the specimen slide, just in front of the blood drop.
- Hold the spreader slide at a 30 angle, and draw it back against the drop of blood.
- Allow the blood to spread almost to the edges of the slide.
- Push the spread forward with one light, smooth, and fluid motion. A thin film of blood in the shape of a bullet with a feathered edge will remain on the slide.
- Label the frosted edge with patient name, ID number and date.

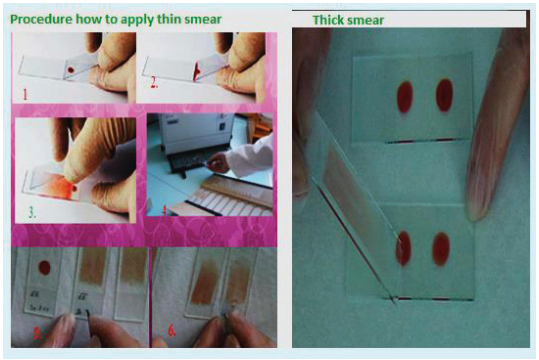

Figure 6: Thin smear and thick smear.

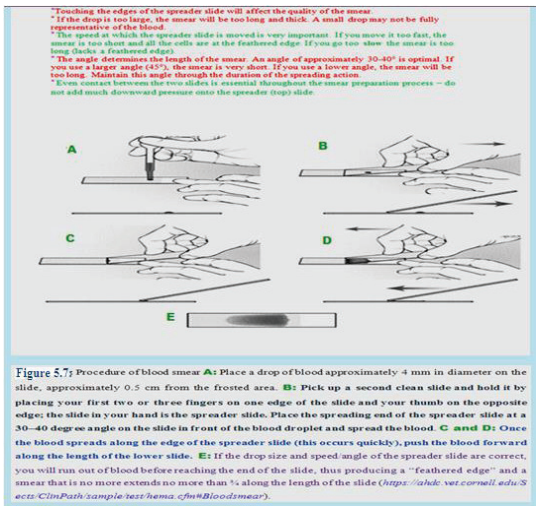

Figure 5.7: Procedure of blood smear **A:** Place a drop of blood approximately 4 mm in diameter on the slide, approximately 0.5 cm from the frosted area. **B:** Pick up a second clean slide and hold it by placing your first two or three fingers on one edge of the slide and your thumb on the opposite edge; the slide in your hand is the spreader slide. Place the spreading end of the spreader slide at a 30–40 degree angle on the slide in front of the blood droplet and spread the blood. **C and D:** Once the blood spreads along the edge of the spreader slide (this occurs quickly), push the blood forward along the length of the lower slide. **E:** If the drop size and speed/angle of the spreader slide are correct, you will run out of blood before reaching the end of the slide, thus producing a "feathered edge" and a smear that is no more extends no more than ¾ along the length of the slide (https://ahdc.vet.cornell.edu/Sects/ClinPath/sample/test/hema.cfm#Bloodsmear).

Figure 7:

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- Allow the blood film to air-dry completely before staining. (Do not blow to dry. The moisture from your breath will cause RBC artifact).

The good blood smear should have the following:

A dense body; this should take up about 2/3 of the entire smear and should blend smoothly into the monolayer area. A well developed feathered edge. This edge should have a fine, feathery appearance; if there is a thick line of blood where the slide stopped, it's an indication of a poorly made smear. A monolayer area just behind the feathered edge. This region should be noticeably thinner than the body, but should blend in with the body of the smear. Often this area is only about ½ cm wide. Prior to staining, if the slide is held up to the light, there is a rainbow effect seen just behind the feathered edge on wellmade smears.

- A good blood film preparation will be thick at the drop end and thin at the opposite end.
- As soon as the drop of blood is placed on the glass slide, the smear should be made without delay. Any delay results in an abnormal distribution of the white blood cells, with many of the large white cells accumulating at the thin edge of the smear.
- The blood smear should occupy the central portion of the slide and should not touch the edges.
- The thickness of the spread when pulling the smear is determined by the 1) angle of the spreader slide (the greater the angle, the thicker and shorter the smear), 2) size of the blood drop and 3) speed of spreading.
- If the hematocrit is increased, the angle of the spreader slide should be decreased.
- If the hematocrit is decreased, the angle of the spreader slide should be increased.

Common causes of a poor blood smear:

- Drop of blood too large or too small.
- Spreader slide pushed across the slide in a jerky manner.
- Failure to keep the entire edge of the spreader slide against the slide while making the smear.
- Failure to keep the spreader slide at a 30 angle with the slide.
- Failure to push the spreader slide completely across the slide.
- Biologic causes of a poor smear:

✓ Cold agglutinin - RBCs will clump together. Warm the blood at 37 oC for 5 minutes, and then remake the smear.

✓ Lipemia - holes will appear in the smear. There is nothing you can do to correct this.

✓ Rouleaux - RBC's will form into stacks resembling coins. There is nothing you can do to correct this.

The WBCs are unevenly distributed and RBC distortion is seen at the edges. Smaller WBCs such as lymphocytes tend to reside in the middle of the feathered edge. Large cells such as monocytes, immature cells and abnormal cells can be found in the outer limits of this area. Spun smears produce the most uniform distribution of blood cells.

Blood staining

Wright's Stain

The Wright's stain is a Romanowsky stain. A Romanowsky stain is any stain combination consisting of eosin Y or eosin B with methylene blue and/or any of its oxidations products. Such stains produce the typical purple coloration of leukocyte nuclei and neutrophilic granules as well as the numerous blues and pinks found in other cell types. Methyl alcohol is used as both a solvent and fixative in this procedure [60-64].

Procedure:

- Attach a clothes pin (or use forceps) to the thick edge of the blood smear.
- Place the slide in the Coplin jar with Wright's stain. Allow to stand 5-10 seconds.
- Raise the slide out of the stain and allow the majority of the stain to run off the slide.
- Place the slide in the first jar containing deionized water. Allow to stand 10-20 seconds.
- Remove the slide carefully and dip several times in the second jar containing deionized water to rinse off the excess stain.
- Wipe off excess fluid from the back of the slide. Place the slide upright on a paper towel with the feathered edge up and allow to air dry.
- When completely dry, examine the smear with the microscope (lower power 10x) as follows:
- **Determine the overall staining quality of the blood smear:**

- Stain should not be too dark or too pale.
- There should be no stain precipitate present on smear.
- RBCs should be appropriate color of reddish pink.
- Lymphocytes have dark purple nuclei with varying shades of blue cytoplasm
- Neutrophils have dark purple nuclei with reddish, granular cytoplasm.
- Monocytes have a lighter purple nucleus with a gray-blue cytoplasm.

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- Eosinophils have bright red/orange granules
- Basophils have dark purple nuclei and granules
- Scan the edges and center of the slide to be sure there are no clumps of RBCs, WBCs or platelets. The interpretation result of bood cell is illustrated in table 4.
- In the blood there is some hemoparasite are observed like diroflaria immitus, parafilaria and some protozoal (Figure 8).

Packed Cell Volume

The examination of the blood film for the qualitative assessment of the blood should include blood smear examination for digression from normal of the cell size, shape, distribution, haemoglobin concentration, colour, and intracellular inclusions 55, 65.Analysis of the collected blood samples was divided into the quantitative and qualitative quantification of the red cell, white cells and platelet parameters. The packed cell volume (PCV) was carried out using the microhaematocrit method while the haemoglobin concentration was carried out using the cyanmethaemoglobin method (Figure 9) [66]. The erythrocyte count was estimated by using the haematocytometer method while the erythrocyte indices (mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration) were calculated using the methods described by [67].

Procedure of Microhematocrit [68]:

* Fill the capillary tube two-thirds to three quarters full with well-mixed, oxalated venous blood or fingertip blood. (For fingertip blood useheparinized tubes, and invert several times to mix.)
* Wipe the excess blood from the outside of the tube. Use plain(anticoagulant free)
* Microhematocrit tubes with anticoagulant blood.
* Push sealing clay into one end of each microhematocrit tube. Seal one end of the tube with clay.

Table 4: The morphological color of blood under staining.

Blood cell	Cytoplasm	Nucleus(chromatin)
Erythrocytes	Yellow to pinkish	Purple
Lymphocytes	Blue	Purplish red
Monocytes	Light blue	Purple
Eosinophil	Yellow to brownish red rods	Light purple
Basophils	Dark purple granules	
Neutrophils	Yellow to brownish red rods	Light purple
Thrombocytes	Grey-blue	Purple

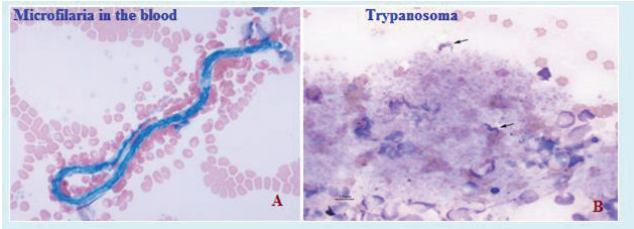

Figure 8: The presence of infectious agent in blood. A: Presence of Dirofilaria immitis (heartworm) or Dipetalonemia-Acanthocheilonema reconditum. B: Trypanosomes (arrows) are caught up within this platelet clump in an anemic dairy coe (htt://www.eclinpath.comd).

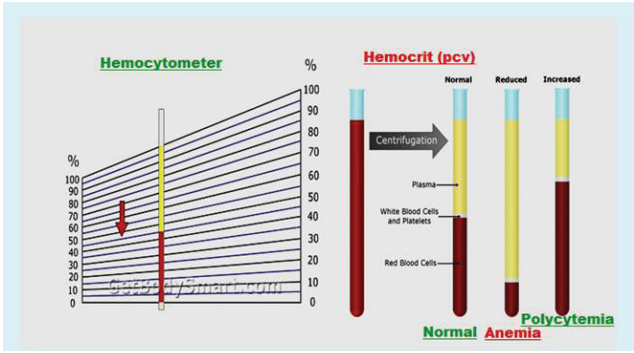

Figure 9: Hemocrit cntrifuge result or PCV and Hemocytometer.

* Place the filled tube in the microhematocrit centrifuge, with the plugged end away from the center of the centrifuge.
* Centrifuge at a preset speed of 10,000 to 12,000 rpm for 5 minutes. If the hematocrit exceeds 50 percent, centrifuge for an additional 3 minutes.
* Place the tube in the microhematocrit reader. Read the hematocrit by hematocrit tube reader.

Hematocrit Value

Hematocri is the percentage of blood volume made up by bed blood cell and it is known as packed cell volume (PCV). It can be deterimined by macro and micro methods. Macromethods are used by Wintrobe tube. With the help of a long needle and syringe, fill the Wintrobe tube with blood upto mark 100. Centrifuge the tubes at 3000 rpm for exactly 30 min. Record the reading of packed cell volume in percent, i.e. mass of erythrocytes settled down in tube. Micromethods is the method use microcapillaries of 1.0 mm diameter and 7–8 cm length are used. The capillaries are filled with blood by capillary action from collection vial. Seal one end of capillary with plasticin and centrifuge in microcentrifuge for 5 min. Remove the capillaries and put them on reader scale to calculate hematocrit value [69].

Packed Cell Volume (PCV) which is also known as haematocrit (Ht or Hct) or erythrocyte volume fraction (EVF) is the percentage (%) of red blood cells in blood [69]. Packed Cell Volume is involvedin the transport of oxygen and absorbed nutrients. Increased Packed Cell Volume shows a better transportation and thus results in an increased

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primary and secondary polycythemia. Haemoglobin is the iron containing oxygen-transport metalloprotein in the red blood cells of all vertebrates with the exception of the fish family, channichthyldae as well as tissues of invertebrates [70]. Haemglobin has the physiological function of transporting oxygen to tissues of the animal for oxidation of ingested food so as to release energy for the other body functions as well as transport carbon dioxide out of the body of animals [71,72]. Packed Cell Volume (PCV) reading indicated either an increase in number of Red Blood Cells (RBCs) or reduction in circulating plasma volume.

Mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration indicate blood level conditions. A low level is an indication of anaemia [73]. The haemotocrit or packed cell volume (PCV) is considered an integral part of an animal's complete blood count result, along with haemoglobin concentration, white blood cell count and platelet count [74,75]. In mammals, haematocrit is independent of body size [75]. The Packed Cell Volume (PCV) can be determined by centrifuging heparinized blood in a capillary tube (also known as microhaematocrit tube) at 10,000 RPM for 5 minutes [76]. This separates the blood into layers. The volume of Packed Red Blood Cells divided by the total volume of the blood sample gives the Packed Cell Volume (PCV). Because a tube is used, this can be measured by measuring the lengths of the layer According to Wikihow (2013c), the Packed Cell Volume (PCV) of animals can be determined to know their anaemia state; the haematocrit is usedto screen animal to determine the extent of anaemia. A low haematocrit combined with other blood abnormal blood tests, confirms the diagnosis. The haematocrit is decreased in a variety of common conditions including liver and kidney diseases, malnutrition, vitamin B12and folic acid deficiencies, iron deficiency, pregnancy among others [76]. A low haematocrit with a low MCV with a high RDW suggests a chronic-iron-deficient anaemia resulting in abnormal haemoglobin synthesis during erythropoiesis [75].

An elevated haematocrit is most often associated with dehydration, which is adecreased amount of water in the tissues, diarrhea etc. These conditions reduce the volume of plasma causing a relative increase in RBCs which concentrates the RBCs, called hemoconcentration [77,78]. Kopp and Hetesa (2000) and Chineke et al. (2006) documented that high PCV haematocrit reading indicated either an increase in the number of circulating RBC or reduction in circulating plasma volume. An elevated haematocrit may also be caused by an absolute increase in blood cells, called polycythemia. This may be secondary to a decrease amount of oxygen, called hypoxia or a result of proliferation of blood forming cells in the bone marrow (Polycythemia vera) [76].

Mean Corpuscular Volume or "Mean Cell Volume" (MCV), is a measure of the average red blood cell volume that is reported as part of a standard complete blood count. The MCV is calculated by dividing the total volume of packed red blood cells (also known as haematocrit) by the total number of red blood cells. The resulting number is then multiplied by 10. The red blood cells get packed together when they are spun around at high speeds in a centrifuge [79,80].

Haemoglobin deficiency can be caused either by decreased amount of haemoglobin molecules, as in anaemia, or by decreased ability of each molecule to bind oxygen at the same partial pressure of oxygen [81]. In any case, haemoglobin deficiency decreases blood oxygen carrying capacity. Haemoglobin deficiency is, in general, strictly distinguished from hypoxemia, defined as decreased partial pressure of oxygen in the blood [81]. Other common causes of low haemoglobin include loss of blood, nutritional deficiency, and bone marrow problems among others. High haemoglobin levels may be caused by exposure to high attitudes, dehydrations, and tumours, among others [82]. The ability of each haemoglobin molecule to carry oxygen is normallymodified by altered blood PH or Co_2, causing an altered oxygen-haemoglobin dissociation curve. However, it can also be pathologically altered in, example carbon monoxide poisoning. If the concentration is below normal, this is called anaemia .Decreased haemoglobin, with or without an absolute decrease of red blood cells, leads to symptoms of anaemia [81]. The Mean Corpuscular Haemoglobin or "Mean Cell Haemoglobin" (MCH) is the average mass of haemoglobin per red blood cell in a sample of blood [83-85]. Mean Corpuscular Haemoglobin Concentration (MCHC) is a measure of the concentration of haemoglobin in a given volume of packed red blood cells. MCHC is very significant in the diagnosis of anaemia and also serve as a useful index of the capacity of the bone marrow to produce red blood cells. It is reported as part of a standard complete blood count. It is calculated by dividing the haemoglobin by the haematocrit [86].

White Blood Cell Count is a test to determine the number of WBCs (Figure 10). Five [87,88] and diverse types of leucocytes exist, but they are all produced and derived from a multipotent cell in the bone marrow known as hematopoietic stem cell. An increase in the number of leucocytes very the upper limits is called leucocytosis and a decrease below the lower limit is called leucopenia. A high white blood cell indicate another problem, such as, infection, stress, inflammation, trauma, allergy, or certain diseases, for this reason, a high white blood cell count requires further investigation 89. High white blood cell count could be caused by infection, immune system disorders, stress, anaemia, bone marrow tumour, infectious diseases inflammatory disease, severe physical stress, tissue damage (for example, burns) among others [89-92]. A low white blood cell count, or leucopenia, is a decrease in disease

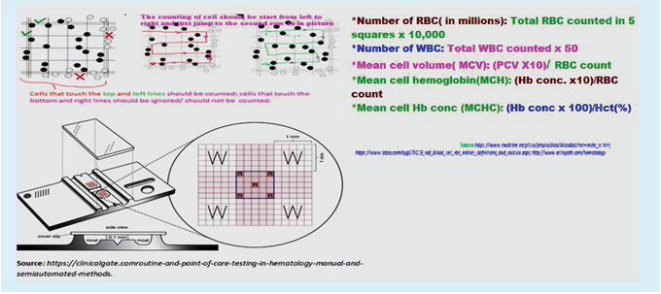

Figure 10: Hemocytometer and calculation of total blood count. The common formual equation which used to calculate the total number of blood cell hemoglobin.

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fighting cells (leucocyte) circulating in animal's body [92,93]. A low number of WBCs may be due to bone marrow deficiency or failure (for example, due to infection,tumour or abnormal scarring), disease of the liver or spleen, radiation therapy or exposure [92-94].

Component of blood smear

Blood smear have several areas while diagnosing under mcicroscope, they are like feathered edge, the monolayer, the body and base of the smear (Figure 11). Among the layer of blood smear monolayer is the best area where the cells are examined in detail and differentiate counted is conducted, due to it dries quickly and cells are well spread (not overlapping) and not disrupted. Red blood cells are separated or barely touching, with little overlapping. In any species but the horse, rouleaux, consisting of stacks of 3 or morered cells, in this area are an abnormality suggestive of increased globulin concentration, which is common in animals with inflammatory disorders [68,95,96].

The animals which have very anemic and polycythemic state, it is difficult to find an area where the RBC are not touching or overlapping. So that the clinician should have to search from feathered edge. The feathered edge of blood smear is the best for detection of platelet clump and some hemoparasite like microflaria, the smear should be examined under lower power microscope. In feathered zone, the RBC is completely flattened and lack of central pallow, mimicking sphereocytes [96,97].

In body zone of blood smear the thick area of a smear dries too slowly for leukocytes to spread out. This area is too thick, however the smear should be examined under low power microscope to detect or identify low number of potentially cells (blast in leukemia), microfilaria, trypanosomes and certain RBC change like hypochromasia and agglutinates (Table 5) [68,97].

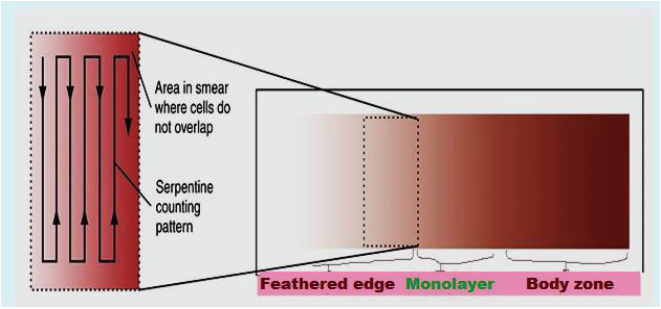

Figure 11: The layer of blood smear.

Table 5: The change of blood cell from examination (http://www.eclinpath.com/hematology).

Blood cell	Shape	Size	Color	Inclusion	Pattern	Infectious Agent
RBC	*Acanthocyte *Echinocyte, *Eccentrocytes *Keratocytes, *Schistocyte *Spherocytes, *Ovalocytes, *Target Cells *Stomatocyte	*Macrocyte, *Microcytes *Anisocytosis (Variation In Size)	*Hypochromasia (Too Little Hemoglobin), *Polychromasia (Immature Cells Containing RNA Or Reticulocytes).	*Siderocytes (Iron), *Basophilic Stippling (RNA), *Howell-Jolly Bodies (Retained Nuclei), *Heinz Bodies (Oxidized Hemoglobin).	*Agglutination (Noted As Present Or Absent), *Rouleaux Formation.	*Babesia, *Anaplasma Spp.
Blood cell	Toxic Change	Reactive Lymphocyte	Smudge Cell	Abnormal Cell	Infectious Agent	
WBC	*Cytoplasmic *Basophilia, *Döhle Bodies, *Cytoplasmic Vacuolation, *Toxic *Granulation, *Immature Chromatin.	Indicating Antigenic Stimulation	A Few Smudged Cells Will Not Impact The Count But Moderate to Many Will. Smudged Cells Are More Frequently Seen In "Aged" Or Stored Samples.	*Presence of Leukemic *Blasts, *Dysplastic *Changes In Leukocytes, Iron In *Leukocytes, *Histiocytes, *Mast Cells.	*Anaplasma,Ehrlichia Spp.	Platelets: Size And Degree of Granularity Is Assessed, Along With The Presence of Anaplasma Sp.

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Citation: Tagesu A (2018) Veterinary laboratory guidance. Int J Vet Sci Res s1: 031-044. DOI: http://dx.doi.org/10.17352/ijvsr.s1.105