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Laboratory Protocols in Animal Physiology Part I: Blood Analysis in Mammals Eliana Pintus, Dott. Ric. and José Luis Ros-Santaella, Ph.D.

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Ai nostri nonni, a nuestros abuelos

Index

How to pipette correctly	7
The blood	9
Blood collection and processing	10
Manual counting of Red Blood Cells (RBCs)	12
Packed Cell Volume (PCV)	16
Hemoglobin (Hb) concentration	19
RBC indices	22
Manual counting of total White Blood Cells (WBCs)	25
Blood smear preparation	27
Blood smear staining	30
Blood smear mounting	31
Blood smear examination	32
Differential counting of WBCs	33
Manual counting of reticulocytes	43
Manual counting of platelets (PLTs)	47
Coagulation tests	49
Erythrocyte Sedimentation Rate (ESR)	51
Determination of human blood groups	55
Blood typing and cross-matching in domestic animals	57

Test	59
Test's solutions	63
List of acronyms	64
References	65
Appendix	66

How to pipette correctly

Pipettes are instruments designed to collect precise volume of liquids or solutions. They are essential instruments for laboratory research studies. Their correct use is extremely important to guarantee that experimental results are accurate and reliable.

There are different types of pipettes. The ones you are going to use during these seminars are single channel and manual micropipettes: "single channel" because only a volume of solution is collected at time, "manual" because the volume of liquid to be collected is manually defined, and "micropipette" because the volume of liquid collected is generally lower than 1 ml (equal to 1,000 μ l). The correct handling of a micropipette is shown in Figure 1.

The volume of liquid that can be collected is shown on the pipette. Some pipettes allow you to collect a range of volume (e.g., 1-10 μ l, 10-100 μ l, and 100-1,000 μ l), whereas others allow you to collect a fixed volume. For adjustable pipettes, the volume can be set by moving the "volume adjustment ring" till the desired position. <u>Never use the pipette for volumes that are outside the pipette's range because it damages the pipette</u>. Once the volume is set, place a clean tip on the tip holder. There are different types of tips according to the type of pipette.

The volume of liquid or solution is collected and released by using the push-button placed on the top of the pipette. By pressing the button, you will feel 2 stops: "first stop" defines the exact desired volume, while the "second stop" (i.e. the deepest one) is only used to release all solution from the tip.

As shown in the Figure 1, you can collect the sample by following these steps:

- 1. Hold the micropipette as shown in Figure 1A. Before placing the tip into the solution, press the push button till the "first stop" position (Figure 1B). By doing so, you will remove the air from the tip. In this way, you will not create bubbles in the solution.
- Keeping the push button pressed in the first position, place the tip into the solution.
 NB: only the top of the tip has to be in contact with the solution!
- 3. Keep the pipette in vertical position and the tip just below the level of fluid. In this way, your accuracy will be relatively high.

4. Smoothly release the press button to allow the volume of solution to move inside the tip.

NB: Avoid making air bubbles and never place the pipette in horizontal position when the tip is filled with solution.

- 5. Discharge the volume in another tube by smoothly pressing the push button till the "second stop" position (Figure 1C).
- 6. Throw away the tip by pressing the tip ejector bottom (the yellow bottom shown in Figure 1).



Figure 1. Correct handling and usage of micropipette. A) correct handling of the micropipette; B) "first stop position" used for collecting a precise volume of solution; C) "second-stop position" used to completely discharge the volume of solution.

The blood

The blood is a type of connective tissue like bones and cartilages. Its main functions include the transport of gases (i.e. oxygen, O₂ and carbon dioxide, CO₂), nutrients, and waste products. The blood is also important for the regulation of the body temperature since it transports the heat from the deepest organs or those showing higher metabolic activity (e.g. brain) and redistributes the heat to the most superficial organs or those displaying lower metabolism (e.g. skin). The blood also transports hormones and clotting factors. Also, it represents a major pH buffer of the organism. The pH of most body fluids is physiologically maintained within pH 7.35-7.45 thanks to the complex buffer system that involves the blood, but also the lungs and the kidneys. The blood osmolality is firmly maintained within 280-300 mOsm/kg H₂O by the kidneys. Values >300 mOsm/kg H₂O (e.g. dehydration) provoke RBC shrinkage (crenation, Figure 2), while values <280 mOsm/kg H₂O provoke RBC swelling and rupture (hemolysis). In general, vertebrates show red-color blood because of hemoglobin (Hb), the respiratory pigment that is responsible for the gas transport. There are few species of Antarctic fish (family Channichthyidae) that surprisingly show colorless blood because they lack of Hb. This trait probably arose as a consequence of the high solubility of O₂ in the cold Antarctic water.

In mammals, the blood represents around 8-10% of body mass and contains 5% of the total body water. Blood is composed of two parts: a liquid fraction (55% of blood volume) and a corpuscular fraction (45% of blood volume). The liquid part is represented by the plasma, which is mainly composed of water, proteins (mostly albumin), electrolytes, hormones, nutrients, and waste products. The corpuscular part is composed of blood elements: cells (erythrocytes, also named red blood cells or RBCs and leukocytes, also named white blood cells or WBCs) and cellular fragments (thrombocytes, platelets or PLTs). In mammals, mature erythrocytes are enucleated, which means that they do not have the nucleus. The mammalian erythrocytes lose the nucleus during the process of erythropoiesis, which mainly occurs in the liver before birth (prenatal stage) and in the bone marrow after birth (postnatal stage). By contrast, in other vertebrates like birds, reptiles, amphibians, and fish, mature erythrocytes and thrombocytes are nucleated.

Blood collection and processing

Blood is generally collected by venipuncture (from jugular, cephalic or saphenous veins) and stored in a tube. There are many types of blood collection tubes (Table 1) according to the type of analysis required. Some analyses require the use of anticoagulants, which are substances that prevents the clotting. The choice of the anticoagulant is based on the type of analysis required. Generally, anticoagulants prevent clotting by chelating calcium (Ca²⁺), which plays a key role during the hemostasis. For the assessment of hemostasis, sodium citrate is commonly used because it is a gentler Ca²⁺ chelator than the ethylenediaminetetraacetic acid (EDTA). Heparin is generally used only for the analysis of biochemical parameters of blood. Heparin is not recommended for blood cells counting or morphological evaluation because it can alter the cell count (i.e. cells tend to clump) and provoke staining artifacts in the blood smears. Interestingly, heparin is the only anticoagulant are usually red-color. They are used for the collection of blood serum and may contain a clot activator.

Type of anticoagulant	Color cap [*]	Type of analysis
EDTA	Purple	CBC; WBC morphology
Heparin	Green	Biochemical parameters tests
Sodium citrate	Blue	Coagulation tests

 Table 1. Common anticoagulants used in veterinary medicine.

^{*}Color cap may change according to the manufacturer. Color cap of blood collection tubes of this table refers to Vacutest Kima s.r.l. (Italy). EDTA: ethylenediaminetetraacetic acid; CBC: Complete Blood Count; WBC: White Blood Cell.

After blood collection, the tube has to be promptly and gently mixed by inversion (blood samples should never be shaken). It is important to correctly fill the collection tube (adequate volume is reported on the tube). Inadequate ratio of anticoagulant/blood volume may alter the analysis. For instance: high ratio of EDTA/blood volume causes cell shrinkage (Figure 2), thus decreasing the packed cell volume (PCV) or the hematocrit (Hct) and the mean corpuscular volume (MCV). On the other hand, low ratio of EDTA/blood volume may not prevent clotting.



Figure 2. Red blood cell (RBC) shrinkage. This artifact may be caused by prolonged sample storage and may give false decrease of PCV and MCV. Porcine blood smear (100× objective).

Blood sample should be analyzed as soon as possible (within 2-3 hours if stored at room temperature in EDTA), otherwise it has to be stored in the fridge. After 6 to 24 hours of storage, RBC swelling may occur, raising the PCV and the MCV while lowering the mean corpuscular hemoglobin concentration (MCHC). Never store the blood samples in the freezer.

Manual counting of Red Blood Cells (RBCs)

The RBCs or erythrocytes (from the Greek *erythros*- red and *–cytos* cell) play a vital role in O_2 transport. They usually have a biconcave disc shape (in goat they tend to be nevertheless roundish). Their diameter ranges from 5 (in goat) to 7 (in dog) μ m. The number of RBCs is important to define the O_2 carrying capacity of blood.

The manual counting of RBCs is intended to determine the number of erythrocytes in the blood. The RBC number is expressed as $10^{6}/\mu l$ or as $10^{12}/l$ (the latter in SI units, see the Appendix). A microliter (μl) is one millionth of a liter (l) or one thousandth of a milliliter (ml).

The principle of manual counting of RBCs can be applied to any other type of cells or particles.

There are different types of counting chambers: Thoma, Fuchs-Rosenthal, Neubauer, and Bürker, just to mention a few. For all of them, the principle is the same; what changes are the area of counting and the depth of the chamber. In this seminar, you are going to determine the RBC number using the Bürker hemocytometer. Each Bürker hemocytometer is composed of two chambers. Because the manual counting is subjective, for each sample RBCs have to be counted on both chambers in order to minimize the error.

Equipment:

- Bürker hemocytometer
- Coverslip
- Hayem's solution (0.25 g HgCl₂, 0.5 g NaCl, 2.5 g Na₂SO₄ and distilled water up to 100 ml, pH 5.9)
- Blood sample with anticoagulant (e.g. EDTA)
- Pipette
- Tips

NB: Hayem solution is toxic because of mercury (Hg). Use gloves when handling the solution and throw it away in appropriate containers.

Procedure:

1. Place a coverslip on the Bürker chamber and fix it with spring clips or little amount of water.

NB: coverslip is fixed when Newton's rings are visible.

- 2. Place $4,975 \ \mu l$ of Hayem's solution in a flask or tube.
- Dilute the blood using the Hayem's solution (25 μl of blood into 4,975 μl of Hayem's solution; Figure 3A).

NB: when pipetting the blood dry the tips externally to avoid miscalculation!

- 4. Gently mix the blood samples at least for three minutes (Figure 3B).
- 5. Pipette 10 μ l of the diluted sample and fill each chamber (keep the chamber in a horizontal plane during and after filling in order to guarantees that cells are homogeneously distributed; Figure 3C).
- 6. Let the RBCs settle down for few minutes.
- 7. Count RBCs in the 20 smallest squares (0.05 mm side; Figure 4).



Figure 3. Procedure for the manual counting of RBCs. A) Blood sample dilution in Hayem's fixative; B) Blood sample mixing; C) Bürker chamber filling.



Figure 4. Bürker counting chamber. Left image shows the grid of the Bürker counting chamber; right image shows the size of the Bürker grid's components.

Per each counting chamber, count the RBCs in the 10 smallest squares (0.05 mm side length; Figure 4).

Count all RBCs inside each square and those ones that touch two sides of the square (Figure 5). **NB: the two sides must be the same through the counting!**

Calculate the total RBC number per each chamber according to the formula:

Number of cells counted \times factor dilution

Area of each square \times number of squares counted \times Depth of the chamber

Number of cells counted: the sum of cells counted in 10 smallest squares per each chamber Factor dilution: 200

Area of each square: $(0.05 \times 0.05) \text{ (mm}^2)$

Number of squares counted: 10

Depth of the chamber: 0.1 (mm)

Check the value of total RBCs per each chamber. The more similar they are, the more repeatable is your counting. If the value of each chamber is similar, calculate the average value of the two chambers.

1. Example:



Figure 5. Representative image of RBC counting. Red dots represent the cells included in the counting because they are located inside the square and on two sides of the square. Blue dots represent cells excluded from the counting.

75: number of cells counted in 10 smallest squares with 0.05 mm side length.

200: dilution factor.

 (0.05×0.05) : area of the smallest square (mm²).

10: number of squares evaluated.

0.1: depth of the chamber (mm).

Check your results in the Table 3 (page 23). In order to calculate the number of RBC per litre, multiply your results by 10^6 (see the Appendix).

Packed Cell Volume (PCV)

The Packed Cell Volume (PCV) and the hematocrit (Hct) represent the percentage of erythrocytes in the blood. The small difference between PCV and Hct concerns the methodological approach: while PCV is evaluated after centrifugation of blood sample in a capillary tube, the Hct is calculated by automated hematology analyzers using the RBC number and MCV values. Both Hct and PCV are important to determine the O_2 carrying capacity of blood. The gross examination of the capillary tube after centrifugation of blood sample also provides information about the color of blood plasma. Abnormal color of blood plasma and serum are shown in Table 2.

Color	Condition	Physiological	Pathological	Artifact
Red	Hemolysis	No	Sepsis, drugs, snake venoms, toxics	Improper sample collection and handling
Dark yellow/green	Icterus (Jaundice)	No	Liver diseases or after hemolytic anemia	No
White	Lipemia	After ingestion of a fatty meal	Pancreatitis, liver diseases, lipid disorders	No

Table 2. Abnormal color of blood plasma/serum.

Equipment

- Micro-hematocrit capillary tube
- Blood sample with anticoagulant (e.g. EDTA)
- Centrifuge
- PCV reading device (centrifuge lid)
- Tissue paper
- Sealing wax

Procedure

- 1. Gently mix the blood sample by inversion.
- Fill the capillary tube with blood up to 4/5 of its length (Figure 6A).
 NB: maintain the capillary tube horizontally and dry it externally with tissue paper (Figure 6B).
- 3. Seal one side of the capillary tube (the one marked by a red ring) with wax (Figure 6C).
- Place the capillary tube into the centrifuge (the sealed side has to be placed externally; otherwise the blood will flow out during centrifugation).
 NB: capillaries tubes have to be placed on the opposite sides to balance the weight within the centrifuge.
- 5. Centrifuge the sample for 3 minutes at 13,000 rpm at room temperature.
- 6. Place the centrifuge lid so that the line corresponding to the value 100 is placed at the top of blood column (plasma, Figure 6D) in the capillary tube. The centrifuge lid works as the reading device.
- 7. Check the value that corresponds to the RBC percentage. Blood fractions can be also clearly observed by the examination of the capillary (Figure 6D). Buffy coat is the whitish line between the plasma and the RBCs and represents the fraction of blood that is mainly composed of WBCs and PLTs.

Check your results in Table 3 (page 23).



Figure 6. Packed Cell Volume (PCV). A) Capillary filling; B) Capillary drying; C) Capillary sealing with wax; D) Blood fractions after centrifugation.

Differences between blood serum and blood plasma:

Blood serum is the liquid fraction of blood after clotting. In other words, the blood serum is the blood plasma without the clotting factors (e.g. fibrinogen).

Blood plasma is the liquid fraction of blood that contains the clotting factors. Blood plasma is obtained by supplementing the blood sample with anticoagulants.

Normally, both plasma and serum show yellowish color.

Hemoglobin (Hb) concentration

Hemoglobin (Hb) is the respiratory pigment responsible for the transport of O_2 in vertebrates. In mammals, a molecule of Hb (64,000-68,000 Dalton) is composed of 4 globins, each one containing a heme group. Each heme group contains an atom of iron at the ferrous state (Fe²⁺). The iron atom <u>reversibly</u> binds to the O_2 forming oxyhemoglobin. The reaction is called oxygenation and it is not an oxidation reaction. Oxyhemoglobin shows bright red color, whereas the O_2 -unloaded form is called deoxyhemoglobin and shows dark-red or bluish color.

Some chemical compounds like nitrate and chloride can oxidize the iron to the ferric state (Fe^{3+}) . The molecule of Hb at this state is called methemoglobin, which is unable to bind O₂ and shows blue color. Physiologically, there is less than 1% of methemoglobin in the mammalian blood. Nitrate poisoning has been reported in cattle grazing on a high fertilized pasture. In dogs and cats, frequent cause of methemoglobinemia is paracetamol administration. Paracetamol is toxic indeed for these species and may cause death. N-acetylcysteine can be used for the treatment of paracetamol toxicity in dogs and cats.

Carboxyhemoglobin has bright red color and is formed when Hb binds to carbon monoxide (CO). The reaction is highly stable because Hb shows more than 200 times more affinity for CO than for O_2 . CO poisoning can occur during exposure to heaters or motor vehicles that run on carbon-based fuels. Hyperbaric O_2 therapy is required in poisoning cases.

The Hb concentration can be determined by spectrophotometry using the Drabkin's reagent. The procedure is based on the oxidation of Hb and its derivatives (except sulphemoglobin) to methemoglobin in the presence of alkaline potassium ferricyanide. Methemoglobin reacts with potassium cyanide to form cyanmethemoglobin, which has maximum absorption at 540 nm. The color intensity is measured at 540 nm and is proportional to the total Hb concentration. Generally, Hb concentration is expressed as g/dl or as g/l (the latter in SI units, see the Appendix).

Equipment

- Pipettes and tips
- Test tubes
- Drabkin's reagent
- Cuvettes (spectrophotometer)
- Spectrophotometer set at 540 nm
- Water bath set at 37 °C

Procedure

- 1. Take 2 clean tubes and label them as S (sample) and B (blank), respectively. Place 7 ml of Drabkin's reagent in each tube (Figure 7A).
- 2. Carefully mix (inversion) the blood and collect 25 μl (<u>NB: when you pipette the blood, dry the tip externally to avoid miscalculation!</u>).
- 3. Place 25 µl of blood into the Drabkin's reagent (Figure 7B).
- 4. Mix by inversion (Figure 7C).
- 5. Place both tubes into the water bath and let them incubate for 20 minutes at 37 °C (Figure 7D).
- 6. Pipette 1 ml of the solution into the cuvette.
- 7. Place the cuvette into the spectrophotometer set at 540 nm and read the sample against the blank (Drabkin's reagent only).

Calculate the Hb concentration according to the formula:

Hemoglobin concentration $(g/dl) = absorbance \times 43$

Check your results in Table 3 (page 23).



Figure 7. Hemoglobin (Hb) concentration. A) Sample (S) and blank (B) tubes containing 7 ml of Drabkin's reagent; B) Sample dilution in 7 ml of Drabkin's reagent; C) Sample mixed by gently inversion; D) Sample and blank incubation for 20 minutes at 37 °C in a water bath.

RBC indices

The RBC indices are calculated based on RBC number, PCV, and Hb concentration. They are important for the classification of anemia, which is a condition characterized by decreased values of RBC number, PCV, and/or Hb concentration. Calculate the RBC indices based on the RBC number, PCV, and Hb concentration recorded on the previous seminars.

Mean Corpuscular Volume (MCV):

The MCV indicates the size of RBC. Low MCV indicates smaller size of RBC, while high MCV indicates bigger size of RBC than normal values.

$$\frac{PCV(\%)}{RBC(10^6/\mu l)} \times 10 = fl$$

The femtolitre (fl) is the metric unit of volume equal to 10^{-15} l or 10^{-9} µl.

Mean Corpuscular Hemoglobin (MCH):

The MCH indicates the Hb content in the RBC. Low MCH indicates smaller amount of Hb in the RBC, while high MCH indicates larger amount of Hb in the RBC than normal values.

$$\frac{Hb (g/dl)}{RBC (10^6/\mu l)} \times 10 = pg$$

The picogram (pg) is the metric unit of mass equal to 10^{-12} kg or 10^{-6} µg.

Mean Corpuscular Hemoglobin Concentration (MCHC):

Similarly to the MCH, the MCHC indicates the Hb concentration in the RBC and is expressed as percentage (or g/l in SI units, see the Appendix).

$$\frac{Hb (g/dl)}{PCV (\%)} \times 100 = \%$$

Variable	Horse	Cattle	Sheep	Goat	Pig	Dog	Cat	Rabbit
RBC number $(10^6/\mu l)$	6-10	5-10	9-15	8-18	5-8	5-8	5-10	5-8
PCV (%)	27-43	24-46	27-45	22-38	36-43	37-57	30-45	33-50
Hb (g/dl)	10-16	8-15	9-15	8-12	10-16	12-19	10-15	10-17
MCV (fl)	37-49	40-60	28-40	16-25	50-68	66-77	39-55	58-67
MCH (pg)	14-18	11-17	8-12	5-8	17-21	21-26	13-17	18-24
MCHC (%)	35-39	30-36	31-34	30-36	30-34	32-36	30-36	29-37

Table 3. Reference values for red blood cells (RBCs) parameters in domestic animals.

RBC: Red Blood Cell; PCV: Packed Cell Volume; Hb: Hemoglobin; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration.

Reference values shown in Table 3 are not segregated based on sex or breed differences. Bear in mind, for instance, that PCV is higher in males than in females (androgens stimulate the erythropoiesis). The hormonal effect of androgens is abolished by castration. There are also breed differences: PCV is higher in hot-blood horses (e.g. Arabian horse) than in cold-blooded horses (e.g. Belgian horse). PCV also differs among dog breeds: for instance, the range value of Hct for St. Bernard is 35-40 %, whereas for Greyhounds is 45-65%. Moreover, especially in horse, PCV can range from 35% at rest to over 60% during race because of spleen contractions.

Causes of increase of RBC number, PCV, and Hb (absolute or relative polycythemia): artifacts (e.g. old samples, RBCs swell increasing PCV and MCV), dehydration, spleen contractions (because of exercise, excitement, and fear, especially in horses), doping (illegal administration of erythropoietin or androgens such as testosterone), and myeloproliferative disorders (absolute polycythemia).

Causes of decrease of RBC number, PCV, and Hb (anemia): blood loss (hemorrhage), bone marrow disease (insufficient production), chronic renal disease (insufficient production of EPO), hemolysis.

Anemias can be classified in several ways based on, for instance, morphological, etiological (etiology means the cause or set of causes of a disease) or prognostic (prognosis means the likely outcome or course of a disease) criteria. The morphological parameters used to classify anemias include the size (i.e. MCV) and Hb content of RBCs. Thus, according to the RBC size (i.e MCV), anemias are classified as normocytic (normal MCV), microcytic (low MCV) or macrocytic (high MCV). On the other hand, based on the Hb content of RBCs (i.e. MCH), anemias are classified as normochromic (normal MCH), hypochromic (low MCH), or hyperchromic (high MCH). Anemias can also be classified as regenerative or nonregenerative based on the capacity of the bone marrow to restore the RBC number. This classification has an important prognostic value: regenerative anemias suggest the beginning of patient's recovery, while non-regenerative anemias do not. The RBC indices are useful to classify the anemia as regenerative or non-regenerative, although other parameters like the percentage and the absolute count of reticulocytes (immature enucleated RBCs) are also important. Non-regenerative anemias are usually normocytic and normochromic (MCV, MCH, and MCHC within the reference values), whereas regenerative anemias are usually macrocytic (higher MCV) and hypochromic (lower MCH). However, it is important to bear in mind that after hemorrhage or hemolysis, the bone marrow requires at least 4-5 days to increase RBC production and show signs of regeneration. Therefore, RBC indices assessed within the 4-5 days after hemorrhage or hemolysis may erroneously be interpreted as nonregenerative anemia. Microcytic (low MCV) and hypochromic (low MCH) anemias are on the other hand frequently related to iron deficiency. Moreover, based on the etiology, the anemias are classified as hypoplastic (insufficient RBC production), hemolytic (excessive RBC destruction), or hemorrhagic (RBC loss).

Manual counting of white blood cells (WBCs)

Leukocytes (from the Greek *leukos*- white and –*cytos* cell) or white blood cells (WBCs) play a key role in the body defense. They are in charge of eliminating pathogens (e.g. virus, bacteria, parasites), infected or neoplastic (i.e. cancer) cells. In the blood, they are less abundant than RBCs. Their number is expressed as $10^3/\mu$ l or as $10^9/l$ (the latter in SI units, see the Appendix). To count the WBCs, the same procedure seen for RBC manual counting is used although with some differences: i) Türk solution is used instead of Hayem solution in order to lyse the RBCs; ii) blood samples are diluted 1:100 instead of 1:200 because WBCs are less concentrated than RBCs; iii) big squares are used for counting instead of small squares for the same reason previously mentioned.

Equipment:

- Bürker hemocytometer
- Coverslip
- Türk solution (3 ml acetic acid solution, 1 ml gentian violet solution in 100 ml of water)
- Blood sample with anticoagulant (e.g. EDTA)
- Pipettes
- Tips

Procedure:

- 1. Place a coverslip on the Bürker chamber and fix it with spring clips or little amount of water. Coverslips is fixed when Newton's rings are visible.
- 2. Place 990 µl of Türk solution in a flask or tube.
- 3. Gently mix the blood by inversion.
- Dilute the blood in the Türk solution (10 μl of blood into 990 μl of Türk solution).
 NB: when pipetting the blood, dry externally the tips to avoid miscalculation!
- 5. Pipette 10 μ l of the diluted sample and fill each chamber (keep the chamber in a horizontal plane during and after filling).

- 6. Let the sample settle down for few minutes.
- 7. Count WBCs in the 10 big squares (0.2 mm side) in each chamber.
- Count all RBCs inside each square and those cells that touch 2 sides.
 NB: 2 sides must be the same through the counting.

Calculated the total WBC number per each chamber according to the formula:

Number of cells counted × factor dilution

Area of each square \times number of squares counted \times depth of the chamber

Check your results in Table 4.

Table 4. White blood cells (WBCs)	number in	domestic	animals.
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WBC number 6-12 4-12 4-8 4-13 11-22	4-8 4-13 11-22 5-14	6-20 5-13

All values are expressed as $10^3/\mu$ l.

Increase of WBC number (leukocytosis): infections (e.g. virus, bacteria or fungi), infestations (e.g. parasites), inflammation, allergic reactions, bone marrow diseases.

Decrease of WBC number (leucopenia): bone marrow diseases, stress, immunodeficiency (e.g. feline immunodeficiency virus).

Blood smear preparation

The evaluation of blood smear is an important component of blood analyses. It often provides useful diagnostic information that cannot be provided by any hematology analyzer.

Equipment:

- Clean microscope slides
- Blood sample with anticoagulant (e.g. EDTA)
- Pipettes
- Tips

Procedure

- Clean the slide and label it by writing the sample ID on the frosted side of the slide (slide A). Use a pencil! Pen labels are easily deleted by solvents like ethanol.
- 2. Gently mix your blood sample.
- Put a small drop (3-4 mm diameter) of blood close to the frosted end of the slide A (Figure 8A).
- Take another microscope slide (slide B), put at approximately 45° grade on your slide A, keep the entire edge of slide B over slide A, draw it back slowly till slide B touches all the blood's drop (Figure 8B).
- 5. Wait till the blood spreads over the whole edge of the slide B (Figure 8C).
- Draw the slide B smoothly and quickly over the entire length of the slide A (Figure 8D).
- The smear should cover ³/₄ of the slide and ends with a "feathered" edge (Figures 8E and 8F). <u>If the smear is not properly done, discard it and make a new one.</u>
- 8. Let the smear dry for at least 24 hours at room temperature. Do not store the smears in the fridge! Store the smears in a box to protect them from the light and the dust.



Figure 8. Blood smear preparation. A) Small drop of blood is placed on a side of a clean microscope slide; B) Place a slide on a 45° grade and moves slowly close to the blood's drop; C) let the drop diffuse throughout the edge of the slide; D) Make a smear by moving the slide till the opposite side of the slide with a smoothly and quick movement; E) Unstained blood smear; F) Stained blood smears. Upper smear is not correct since it is too thick and lack of "feathered edge", while lower one is correct.

The presence of "feathered" edge in the blood smear is important because in this area the cells are distributed in a monolayer. Morphological evaluation of cells can be done only where they are distributed in a monolayer.

Common causes of poor blood smear quality

- Dirty microscope slide results in uneven distribution of cells.
- Too large or too small drop of blood results in too short or too long smears, respectively.
- Jerky smears are due to improper handling of slide B over the slide A.
- Uneven distribution of cells can be due to the delay in the smear preparation. As soon as the drop is placed on slide A, slide B should be placed over the drop and the smear done.
- Improper angle of slide B: smaller angle results in longer smear; greater angle in shorter smear.
- Failure to keep the entire edge of slide B over slide A during the smear preparation results in uneven cell distribution.

Blood smear staining

Blood smears should be air-dried at least 24 hours at room temperature before staining.

Equipment:

- Labeled blood smears
- Staining solutions (e.g. Leucodiff 200[®])
- Washing solution
- Coplin jar

Procedure:

- 1. Dip the smear 5 times (1 second each) in reagent 1 (fixative).
- 2. Drip off well the excess of fixative on a filter paper to limit cross-contamination of solution.
- 3. Dip the smear 5 times (1 second each) in reagent 2 (acid solution, red color).
- 4. Drip off well the excess of reagent 2 on a filter paper to limit cross-contamination of solution.
- 5. Dip the smear 6 times (1 second each) in reagent 3 (basic solution, blue color).
- 6. Drip off well the excess of reagent 3 on a filter paper to limit the contamination of washing solution.
- 7. Rinse for few seconds with distilled water.
- 8. Air dry in vertical position for 15-20 minutes and then store in a box for at least 24 hours at room temperature before mounting.

Blood smear mounting

Equipment:

- Labelled, stained and air-dried blood smears
- Pasteur pipette
- Mounting medium (e.g. DPX, Entellan, Neo-Mount)
- Xylene
- Coverslip (24×60 mm)
- Laminar flow cabinet

Procedure:

- 1. With the aid of a Pasteur pipette, put a drop of mounting medium on a coverslip.
- 2. Embed the smear for a second in the xylene solution and drop the excess on the mounting medium over the coverslip.
- 3. Place the blood smear over the coverslip avoiding the formation of air bubbles in the mounting medium.
- 4. Put the mounted smear in horizontal position on a paper tray and let it dry for at least 24 hours at room temperature.

Blood smear examination

Blood smear examination can be applied to evaluate different parameters:

- 1. **Differential WBC counting:** this analysis is useful to provide the percentage of WBC types. Absolute number of a WBC type can be estimated by multiplying the percentage by the total WBC number.
- 2. WBC morphology: for instance, toxic changes of neutrophils because of inflammation.
- 3. **RBC morphology:** abnormal RBC morphology because of improper storage (crenation) or pathological conditions.
- 4. **PLT morphology and counting:** total platelets number can be estimated on a well-made blood smear. The degree of granulation and presence of PLT clumps can be also observed.
- 5. Pathogens: Plasmodium spp., Leishmania spp., Babesia spp., Toxoplasma gondii, and microfilariae, etc.

Irrespective of the purpose of the analysis, blood smears are usually evaluated using the battlement method (Figure 9) in order to not count the same cell or microorganism twice.



Figure 9. Blood smear evaluation by battlement method.

Differential counting of WBCs

The manual WBC differential count consists on classifying 100 WBCs on a blood smear by bright field microscopy ($100 \times$ objective, oil immersion). The aim of this analysis is to determine the percentage of each type of WBC.

Since the analysis is performed under $100 \times$ objective (oil immersion), the smears have to be previously mounted with a coverslip.

Equipment:

- Mounted blood smear
- Oil immersion
- Microscope

Procedure:

- 1. Switch on the microscope and place the blood smear on the microscope stage.
- 2. Focus the cells using the low magnification objectives $(10\times, 20\times, \text{ and then } 40\times)$.
- Place a small drop of immersion oil on the smear and switch to the 100× objective NB: immersion oil is used for the 100× objective only.
- 4. Analyze the blood smear on the "feathered edge" where the cells are distributed in a monolayer.

NB: A consistent pattern must be used to avoid counting the same cell twice (Figure 9).

5. After finishing the analysis, clean the objective and the smear's coverslip with tissue paper embedded with solvent like isopropyl alcohol.

Based on the morphology of the cytoplasm and the nucleus (Figure 10), WBCs are classified as:

- **Granulocytes** or **polymorphonuclear leukocytes**: they show granules in the cytoplasm and a multilobed nucleus.
- Agranulocytes or mononuclear leukocytes: they do not show neither granules in the cytoplasm nor lobed nucleus.



Figure 10. White blood cells (WBCs). Agranulocytes or mononuclear leucocytes (*left*) and granulocytes or polymorphonuclear leukocytes (*right*). Porcine blood smear $(100 \times \text{ objective})$.

Moreover, based on the staining of the cytoplasmic granules, granulocytes or polimorphonuclear leukocytes are classified as:

- Neutrophils: multilobed nucleus with colorless granules (Figure 11).
- **Eosinophils**: bilobed nucleus with pink/orange granules (some examples <u>here</u>)
- **Basophils**: bilobed nucleus (often obscured by cytoplasmic granules) with purple/blue granules (Figure 12).



Figure 11. Neutrophil. Porcine blood smear (100× objective).



Figure 12. Basophil. Porcine blood smear (100×objective).



Figure 13. Monocyte. Porcine blood smear (100× objective).



Figure 14. Large (left) and small (right) lymphocytes. Porcine blood smear (100× objective).

Agranulocytes or mononuclear leukocytes are further classified as:

- **Monocytes**: large cells with abundant cytoplasm and pleomorphic (bean-shaped) nucleus, often eccentrically located (Figure 13). Vacuoles may be observed within the cytoplasm. Cell edges might be irregular.
- Lymphocytes: round cells with large, medium, or small size. The cytoplasm is very scarce compared to the size of the nucleus, in other words they have a small nucleus/cytoplasm ratio (Figure 14).

Count 100 WBCs and classify them as indicated above. Check your results in Table 5.

Variable	Horse	Cattle	Sheep	Goat	Pig	Dog	Cat	Rabbit
Neutrophils	52-70	15-33	10-50	30-48	28-47	58-85	45-64	20-75
Lymphocytes	21-42	45-75	40-55	50-70	39-62	8-21	27-36	30-85
Monocytes	0-6	0-8	0-6	0-4	2-10	2-10	0-5	1-4
Eosinophils	0-7	0-20	0-10	1-8	0-11	0-9	0-4	1-4
Basophils	0-2	0-2	0-3	0-1	0-2	0-1	0-1	1-7

Table 5. Percentage of WBCs in domestic animals.

Neutrophils' percentage refers to the segmented neutrophils. Segmented neutrophils are mature neutrophils that differ from the immature counterpart called band neutrophils. Band neutrophils usually increase after inflammation as a response of bone marrow stimulation. Band neutrophils are not illustrated here because the topic is beyond the scope of this manual.

Based on the WBC manual count, you can also estimate the absolute number of each WBC type by multiplying the percentage per the total WBC count. The percentage of WBC population is meaningless if not compared to the total number of WBC types.

Check your results in Table 6.

Neutrophils and lymphocytes are physiologically the most abundant WBCs, although there are differences between species. In rabbits and in adult ruminants and pigs, lymphocytes are more abundant than neutrophils. By contrast, in horses, dogs, cats, and in young ruminants and pigs, neutrophils are more abundant than lymphocytes.

Therefore, the ratio between neutrophils and lymphocytes is physiologically >1 in horses, dogs, and cats, and <1 in rabbits and in adult ruminants and pigs. Variations from the normal values in healthy individuals may be indicative of stress.

Variable	Horse	Cattle	Sheep	Goat	Pig	Dog	Cat	Rabbit
Neutrophils	2.9-8.5	0.6-4	0.7-6	1.0-7.2	2-15	2.9-12	2.5-12.5	1-9.4
Lymphocytes	1.2-5.1	2.5-7.5	2-9	2-9	3.8-16.5	0.4-2.9	1.5-7	1.6-10.6
Monocytes	0-0.7	0-0.9	0-0.8	0-0.6	0-1	0.1-1.4	0-0.9	0.1-0.5
Eosinophils	0-0.8	0-2.4	0-1	0.1-0.7	0-1.5	0-1.3	0-0.8	0.1-0.5
Basophils	0-0.3	0-0.2	0-0.3	0-0.1	0-0.5	0-0.1	0-0.2	0.1-0.9

 Table 6. WBC number in domestic animals.

All values are expressed as $10^3/\mu$ l. Neutrophil number refers to the segmented neutrophils. Segmented neutrophils are mature neutrophils that differ from the immature counterpart called band neutrophils. Band neutrophils usually increase after inflammation as a response of bone marrow stimulation. Band neutrophils are not illustrated here because the topic is beyond the scope of this manual.

Role of WBCs and causes of the alterations of WBC types:

Neutrophils

They are the first line of defense and show high phagocytotic activity. When they phagocytose pathogens, they die and often result in the formation of pus. They live for few days and when they leave the blood vessels (diapedesis), they do not come back.

Causes of *neutrophilia* (increased number of neutrophils): infections, inflammation, and high levels of endogenous or exogenous corticosteroids (stress leukogram pattern). Neutrophilia may be induced by high levels of epinephrine as a physiological response to acute stress (frequent in cats and rabbits) or strenuous exercise (frequent in horse).

Causes of *neutropenia* (decreased number of neutrophils): decreased production or release from the bone marrow, acute inflammation (in cattle since they do not have great pool reserve of neutrophils), viral diseases (bovine virus diarrhea, canine parvovirus, feline leukemia, and feline immunodeficiency virus), drugs, and toxins.

Eosinophils

Eosinophils are mainly involved in allergic reactions and parasite infestations. They live for few days, show ameboid movement, and at least to some extent phagocytotic activity.

Causes of *eosinophilia* (increased number of eosinophils): parasitic infestations and allergic reactions.

Causes of *eosinopenia* (decreased number of eosinophils): sometimes associated to inflammation or stress leukogram in response to high levels of endogenous or exogenous corticosteroids.

Basophils

Basophils are rarely observed in blood smears. Their characteristic blue cytoplasmic granules contain heparin (anticoagulant) and histamine, among others. Histamine relaxes smooth muscles of blood vessels and contracts smooth muscles of airways. By doing so, histamine lowers the blood pressure (hypotension) while makes breathing more difficult (bronchoconstriction). They are involved in allergic reactions and anaphylactic shock.

Causes of *basophilia* (increased number of basophils): rare finding usually associated with eosinophilia. In absence of eosinophilia, it is associated with bone marrow disorders.

Causes of basopenia (decreased number of basophils): no clinical significance.

Monocytes

Monocytes are the largest leukocytes with high phagocytotic activity. They play a major role in the regulation of immune response. When they leave the blood vessels, they become macrophages. Once they leave the blood vessels, they do not return. They can live for several months.

Causes of *monocytosis* (increased number of monocytes): chronic inflammation, infections, and high levels of corticosteroids (stress leukogram, quite frequent in dogs).

Causes of monocytopenia (decreased number of monocytes): no clinical significance.

Lymphocytes

Lymphocytes are important in the regulation of long-lasting defense (immune memory) and play a major role against cancer cells. They can live for years and can leave/re-enter the blood vessels several times during their lifetime.

Causes of *lymphocytosis* (increased number of lymphocytes): infections, but also physiological in young animals (usually <6 months old). It can also be provoked by acute stress because of fear or excitement (in cats, rabbits, and horses) and by strenuous exercise (in horses).

Causes of *lymphopenia* (decreased number of lymphocytes): usually associated with prolonged stress because of high levels of endogenous cortisol (stress leukogram) or exogenous corticosteroids.

Manual counting of reticulocytes

Reticulocytes are immature and enucleated erythrocytes. Their name is due to the presence of a reticular network of ribosomal RNA that becomes visible using specific staining (e.g., new methylene blue, NMB). Mature erythrocytes usually contain very low amount or no RNA. The number of reticulocytes is normally low, but during anemia their number increases as a sign of regenerative response by the bone marrow, especially in dogs and cats. Compared to mature RBCs, reticulocytes are generally larger (macrocytic) and contain relatively lower amount of Hb (hypochromic). For this reason, regenerative anemia is usually associated with high MCV and low MCH and MCHC. The reticulocytes counting is performed whenever the PCV $\leq 20\%$ in cats and $\leq 30\%$ in dogs. The horse is an exception because the number of reticulocytes is always very low even during anemia.

Based on the RNA content and appearance, there are two types of reticulocytes: aggregate reticulocytes and punctate reticulocytes. Aggregate reticulocytes are more immature than punctate reticulocytes. The lifespan of these two stages of reticulocytes' development is similar between species, except in the cat. In cats, aggregate reticulocytes last 12-24 hours, while punctate reticulocytes last between 7-10 days. Therefore, in this species the count of aggregate reticulocytes is used to assess the regenerative anemia. Feline punctate reticulocytes show 3-12 dots, while aggregate reticulocytes show more than 12 dots. In dogs, punctate reticulocytes are not frequent. Therefore, the classification of reticulocytes is not required in this species.

Reticulocytes counting can be performed manually or using automated equipments. Obviously, manual counting is more imprecise than automated counting. However, no automated equipment is able to distinguish aggregate from punctate reticulocytes. Therefore, in cats, counting of aggregate and punctate reticulocytes can be done only manually.

Equipment:

- Blood sample with anticoagulant (EDTA)
- NMB dye: 1 gr of NMB dissolved in 100 ml of solution composed of 3 gr sodium citrate and 0.9 gr NaCl. Filter once the dye is dissolved.
- Pasteur pipette
- Microcentrifuge tubes
- Microscope slides and coverslips
- Microscope

Procedure:

- 1. Put 2 drops of blood sample into a microcentrifuge tube. Then add 2 drops NMB dye.
- 2. Mix and incubate at 37 °C for 20 minutes.
- 3. Gently mix the cells and make a blood smear.
- 4. Let the smear air dry.
- 5. Mount the smear with a coverslip.
- 6. Examine under 100× objective.
- 7. Determine the percentage of reticulocytes in 1,000 mature RBCs. Mature RBCs are unstained because they lack RNA.

Regenerative anemia is characterized by >1% reticulocytes in dogs, >0.4% in cats.

However, the percentage of reticulocytes alone is misleading because in anemia RBCs are lower than normal. Therefore, the percentage of reticulocytes may overestimate the bone marrow response. The absolute reticulocytes count is obtained on the basis of the RBC number.

Absolute reticulocyte count

Absolute reticulocyte count is obtained by multiplying the percentage of reticulocytes by the RBC number. Actually, the absolute reticulocyte count is the criterion used to classify anemia as regenerative or non-regenerative in dogs and cats.

Table 7. Reticulocytes percentage and absolute count in dogs and cats.

Variable	Dog	Cat
Reticulocytes (%)	0-1.0	0-0.6
Absolute reticulocyte count $(10^3/\mu l)$	<80	<60

To understand the importance of estimating the reticulocyte absolute count with respect to the percentage of reticulocytes, three clinical cases in feline patients are shown in Table 8.

	PCV (%)	RBC no. (10 ⁶ /µl)	Reticulocytes (%)	Reticulocyte absolute count $(10^3/\mu l)$
Cat 1	35	8	0.4	32
Cat 2	25	4	1.6	64
Cat 3	20	3	2	60

Table 8. Examples of different reticulocyte percentage and absolute count in feline patients.

PCV: Packed Cell Volume; RBC: Red Blood Cell; no: number.

Cat 1 is healthy and show normal values of PCV, RBC number, and reticulocytes' percentage and absolute count. On the other hand, Cat 2 and cat 3 are anemic (PCV <30% and RBC number $<5 \times 10^6/\mu$ l). Although Cat 3 shows higher percentage of reticulocytes, the reticulocyte absolute count is only 60,000/µl. By contrast, Cat 2 shows both higher percentage and absolute count of reticulocytes. These results indicate that Cat 3 shows signs of nonregenerative anemia, while Cat 2 shows signs of regenerative anemia. The prognosis for Cat 3 is therefore less optimistic than the one for Cat 2.

Manual counting of platelets (PLTs)

In mammals, thrombocytes (from the Greek *thrombos*– clot and –*cytos* cell), also named platelets (PLTs), are cellular fragments (enucleated) that originate from megakaryocytes in the bone marrow (Figure 15). They are the smallest blood elements, ranging in size from 2 to 4 μ m. The PLTs play a crucial role in blood clotting. Manual counting of PLTs can be done using a hemocytometer, although it is often difficult to distinguish PLTs from debris, dust or scratches. PLT number is expressed as 10⁵/µl or as 10⁹/l (the latter in SI units, see the Appendix).



Figure 15. Platelets (PLTs). Porcine blood smear (100× objective).

Alternatively, PLTs can be manually counted on a well-made blood smear. Blood smear has to be prepared using blood samples supplemented with EDTA. Heparin indeed causes platelets clumping. The PLTs have to be counted on a monolayer in 5 to 10 fields under a $100 \times$ oil immersion objective. In general, dogs show 8 to 29 PLTs per field, while cats show 10 to 29 PLTs per field. In patients affected by severe thrombocytopenia (low PLT number) 0 to 3 platelets are observed per each field. It should be taken into account that the estimated manual counting of platelets is rather difficult in the cats because their platelets tend to clump

very easily. PLTs clump also during storage. Therefore, manual PLT counting should be performed within 6 hours after blood collection. In sample where PLTs clump, estimation of PLT number is inaccurate.

Total number of PLTs per μ l can be roughly estimated according to the formula:

mean number of PLTs per field \times 15,000

Check your results in Table 9.

Table 9. Number of	platelets ((PLTs)) in (lomestic	animal	s.
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Variable	Horse	Cattle	Sheep	Goat	Pig	Dog	Cat	Rabbit
PLT no.	117-256	100-800	800-1,100	300-600	200-500	211-621	300-800	250-650
All values are expressed as $10^3/\mu$ l. PLT: platelet.								

Causes of the alterations of PLT number:

Causes of *thrombocytosis* (increased number of PLTs): bone marrow diseases (myeloproliferative disorders).

Causes of *thrombocytopenia* (decreased number of PLTs): artifact (long sample storage or PLT clumping), bone marrow disorders, and increased consumption in the coagulation process or destruction by macrophages. The Cavalier King Charles spaniels often are affected by a benign hereditary PLT disorder: individuals from this canine breed frequently show lower number ($<10,000/\mu$ l) but larger size of platelets with no symptoms (e.g. bleeding).

Coagulation tests

Hemostasis is a complex process that limits the bleeding, thus blood loss. Several components are involved: PLTs, endothelial cells, minerals, clotting factors, and clotting inhibitors. Therefore, several tests are designed to identify the defects of the coagulation process.

The hemostasis is generally classified in three phases: primary hemostasis, secondary hemostasis, and fibrinolysis. These phases do not occur sequentially but rather simultaneously, although at different degrees.

Primary hemostasis entails the formation of the PLT plug. It involves PLTs, endothelial cells, collagen, adenosine diphosphate (ADP), and thromboxane (both ADP and thromboxane are released by PLTs). After vessel injury, sub-endothelial collagen is exposed. Then, the PLTs switch from the resting state to the activated state, which consists on the formation of pseudopods and release of granules' content.

Secondary hemostasis concerns the formation of fibrin clot. The phase is organized in extrinsic and intrinsic pathways, which both converge into the common pathway. The pathways consist on complex cascade reactions involving Ca^{2+} and several clotting factors, which finally activate the prothrombin to thrombin. Then, thrombin converts the plasma fibrinogen into fibrin, resulting in the clot stabilization.

Fibrinolysis concerns the clot removal. This phase mainly involves the endothelial cells and plasminogen. Plasminogen is converted to plasmin by the tissue plasminogen activator, among others, which degrades the fibrin and breaks down the clot.

Here, we illustrate two tests that can be easily performed in a veterinary clinic. Nevertheless, both tests are neither specific nor sensitive, although they can suggest some coagulation diseases. Other more sensitive and precise tests are for instance: the prothrombin time, the activated partial thromboplastin time, and the thrombin clot time.

Capillary bleeding time

This test identifies defects in the primary hemostasis (clot formation). It is an easy though subjective *in vivo* test. The capillary bleeding time is performed on the lateral surface of the front toe after skin shaving and disinfection. The animal is kept on a lateral recumbency during the procedure. The test can be also performed on the buccal mucosa of the upper lip. The mucosa is secured by gauze tied around the maxilla (the gauze must be no too tight-fitting). In both cases, a cut is performed using a bleeding device provided by a lancet, which is specifically designed for this test (the cut is usually 1 mm deep). The excess of blood should be carefully removed using a filter paper without touching the site of cutting. The bleeding stops within 3 minutes in healthy individuals. Longer bleeding time may indicate low PLT number and impaired function, von Willebrand's disease (frequent in Doberman), or vascular diseases (rare).

Activated coagulation time

Blood is collected with a sterile syringe and placed in a pre-warmed (37 °C) activated coagulation time tube. Then, the sample is incubated at 37 °C in a water bath for 1 minute in dogs or 45 seconds in cats. After that, the sample is gently inverted once every 5 seconds. The activated coagulation time is the interval of time from the beginning of incubation till the formation of the first clot. The clot is generally completely formed within 110 seconds in dogs and within 75 seconds in cats. However, the reference values differ depending on the type of the activator used. Longer activated coagulation time may indicate low PLT number or ingestion of warfarin-type rodenticides.

Erythrocyte Sedimentation Rate (ESR)

The test consists on measuring the rate at which the RBCs sediment in a period of time. In veterinary practice this test is not commonly used because it is also extremely species-specific. It is a sensitive (because it determines the presence) but not specific (it does not determine the site or the agent) test to detect inflammation. It may also have some prognostic value (it may indicate if the patient is recovering from illness or not).

Inflammation is one of the innate first-line defenses of the immune system. It can be defined as a pathophysiological condition characterized by the following features:

- Pain (Dolor)
- ✤ Heat (Calor)
- Redness (*Rubor*)
- Swelling (*Tumor*)
- Loss of function (*Functio lesa*)

The inflammation can occur after insect's sting, bone fracture, burns, infections, etc.

During inflammation, the ESR increases because proteins released by the immune system (mainly fibrinogen and gamma globulins) change the plasma viscosity, making RBCs agglutinate and sediment quicker than normal.

Equipment (Westergren method):

- ESR tubes (20 mm height)
- Blood sample with anticoagulant (sodium citrate)

Procedure:

- 1. Gently mix the blood sample by inversion.
- 2. Fill the cuvette till the mark avoiding bubbles (Figure 16A and B).

- 3. Insert the tube in the cuvette till the blood reaches the value 0 in the tube (Figure 16C).
- 4. Keep the tube in horizontal position during the whole incubation time, which varies according to the species (see Table 10).
- 5. Check the mm of sedimentation as shown in Figure 17.



Figure 16. Erythrocyte sedimentation rate (ESR). A) and B) cuvette filling till the mark; C) tube insertion and filling till the value of 0 mm.

Species	10 min	20 min	30 min	1 h	24 h
Horse	2-12	15-38			
Cattle					2-4
Sheep					3-9
Goat					2-3
Pig			0-6	1-14	
Dog			1-6	5-25	
Cat				7-23	

Table 10. Reference values for ESR in domestic animals.

Values are shown as mm of sedimentation.



Figure 17. Erythrocyte sedimentation rate (ESR). Porcine blood sample before A) and after B) incubation. ESR after 1h of incubation is 2 mm, thus within the normal range for this species.

NB: The tendency of equine RBCs to form rouleaux makes them to sediment very fast. On the other hand, ruminants' RBCs tend to sediment very slowly.

Factors affecting the ESR:

Environmental: Temperature (i.e. high temperature increases the ESR).

Physiological: Pregnancy (increases the ESR), high PCV or Hct (increase the ESR).

Pathological: Inflammation, infection, cancer, pathological increase of PCV.

Determination of human blood groups

Blood groups are defined based on the presence of antigens on the RBC plasma membrane. There are 35 groups in human blood. The most important are the AB0 system and Rh system.

Equipment:

- Clean microscope slides and coverslips
- Sterile needles
- Disinfectant
- Tissue paper
- Patches
- Antibodies against AB0 and Rh systems



Figure 18. Determination of human blood groups.

Procedure:

- Put a drop of each antibodies solution on a microscope slides (put in order solutions against A antigens (Anti-A), B antigens (Anti-B), AB antigens (Anti-AB), and Rh antigen (Anti-D), respectively). Each drop must not be mixed with others (Figure 18A).
- 2. Clean and disinfect the tip of your index finger.

- 3. Sting the tip of your index finger with a sterile needle and let the blood come out by making pressure close to the wound.
- 4. With the corner of the coverslip take a little amount of blood and mix it with the solution against A antigens.
- Change the corner of the coverslip and repeat the same procedure with solution against B, AB, and Rh antigens (Figure 18B).
 NB: use a clean corner of the coverslip per each solution.
- 6. Clean your wound and place a patch on the puncture site.
- 7. Let the samples incubate for few minutes. Then, observe if there is or there is not agglutination. Agglutination occurs when RBCs clump. This phenomenon is due to the interaction between the antigens found on the RBC plasma membrane and the antibodies in the solution. Determine the blood group as shown in Figure 19.



Figure 19. Human blood groups.

Blood typing and cross-matching in domestic animals

Blood groups differ among species. Blood typing in dog is complex because there are many blood groups, while in cats there are only three groups: A, B, and AB (the latter is very rare). Short-hair cats are usually type A, while some breeds (British short-hair, Devon rex, and exotic cats) are usually type B.

To avoid life-threatening situation caused by transfusion rejection, it is very important to cross-match the blood from the donor and the recipient. The principle is to see if the recipient has antibodies against the donor (major cross-match).

Equipment:

- Blood sample (with anticoagulant) from the donor and the recipient
- Physiological solution
- Pipettes and tips
- Tubes
- Centrifuge
- Water bath set at 37 °C

Procedure:

- 1. Label 2 tubes with donor and recipient, respectively.
- 2. Collect 2 ml of blood donor and put in the tube. Repeat the same procedure for the blood recipient.
- 3. Centrifuge at 2,500 rpm per 3 minutes.
- 4. Aspirate the donor's blood plasma and put in a separate tube. Repeat the same procedure for the recipient's blood plasma.
- Resuspend the pellet (RBCs suspension) with 4 ml of physiological solution (0.9% NaCl).
- 6. Centrifuge at 2,500 rpm per 3 minutes.

- 7. Remove the supernatant. Repeat step 5 and 6 three times.
- 8. Then, combine 1 drop of RBCs with 2 drops of plasma as follows:
 - Donor's RBCs + Recipient's plasma = major cross-match.
 - Recipient's RBCs + Donor's plasma = minor cross-match.
 - \blacktriangleright Recipient's RBCs + Recipient's plasma = control tube.
- 9. Mix and incubate at 37 °C per 15 minutes.
- 10. Centrifuge at 2,500 rpm per 3 minutes. Note if there is hemolysis.
- 11. Resuspend the pellet and observe one drop from each tube. Agglutination indicates the incompatibility between the donor and the recipient. If the recipient shows autoagglutination in the control tube, there were some mistakes in the laboratory procedure. Thus, we cannot determine if the agglutination observed between the donor and the recipient is due to incompatibility between them or to any laboratory mistake. In this case, all procedure has to be repeated.

Test

1. Identify the following blood cells:



- 2. What is the normal color of blood plasma?
 - a) Red
 - b) White
 - c) Yellow
 - d) Green
- 3. Which one of the following factors or conditions may cause neutrophilia?
 - a) Light exercise
 - b) Sexual steroids
 - c) Infection
 - d) Anemia

- 4. Which factor can increase the ESR?
 - a) Low environmental temperature
 - b) Pregnancy
 - c) Low PCV
 - d) High PLT number
- 5. Which is on average the physiological range of PCV across mammals?
 - a) 5-25%
 - b) 25-50%
 - c) 50-75%
 - d) 75-90%
- 6. Among these dog breeds, which one physiologically shows the highest PCV?
 - a) Labrador retriever
 - b) Beagle
 - c) Yorkshire terrier
 - d) Greyhound
- 7. Which type of tissue is the blood?
 - a) Connective
 - b) Epithelial
 - c) Muscular
 - d) Nervous
- 8. Among these factors, which one is NOT associated with the increase of the Hct or PCV:
 - a) Dehydration
 - b) High blood levels of EPO
 - c) High blood levels of testosterone
 - d) Blood loss or hemorrhage

- 9. A white color of blood plasma can be physiologically observed:
 - a) In ruminants
 - b) Early in the morning
 - c) After a meal
 - d) Fasting

10. Regenerative anemia is characterized by:

- a) Low MCV, low MCHC, decreased number of reticulocytes
- b) High MCV, low MCHC, increased number of reticulocytes
- c) Normal MCV and MCHC, increased number of reticulocytes
- d) High MCV, MCHC, and number of reticulocytes
- 11. Which phenomenon is observed if a RBC is placed in a hypertonic solution (>300 mOsm/kg H₂O):
 - a) First crenation, then swelling
 - b) First swelling, then crenation
 - c) Just swelling
 - d) Just crenation

12. In mammals, the RBC number is generally expressed as:

- a) $10^{6}/\mu$ l
- b) 10⁶/1
- c) $10^{3}/\mu l$
- d) $10^{3}/l$

13. After RBC counting using a hemocytometer, which improper procedure may cause a false increase of RBC number:

- a) Blood sample was not properly mixed
- b) Tip was not externally cleaned from excess of blood
- c) Hemocytometer was filled on an oblique position
- d) All the above options may cause a false increase of RBC number

14. Identify the following WBCs:



15. In which species, neutrophils are more abundant than lymphocytes in adulthood:

- a) Cat
- b) Pig
- c) Cattle
- d) Sheep

Test solutions

- 1. A: PLTs; B: neutrophil; C: Limphocyte (left) and neutrophil (right); D: monocyte. RBCs are also observed in all pictures.
- 2. C
- 3. C
- 4. B
- 5. B
- 6. D
- 7. A
- 8. D
- 9. C
- 10. B
- 11. D
- 12. A
- 13. D
- 14. A: basophil; B: lymphocyte; C: neutrophil; D: lymphocyte (above) and neutrophil (below).
- 15. A

List of acronyms

ADP: Adenosine diphosphate

EPO: Erythropoietin

Hb: Hemoglobin

Hct: Hematocrit

MCH: Mean Corpuscular Hemoglobin

MCHC: Mean Corpuscular Hemoglobin Concentration

MCV: Mean Corpuscular Volume

NMB: New methylene blue

PCV: Packed Cell Volume

PLT: Platelet

RBC: Red Blood Cell

RNA: Ribonucleic acid

SI: International System of Units

WBC: White Blood Cell

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Appendix

Variable	Horse	Cattle	Sheep	Goat	Pig	Dog	Cat
RBC number $(10^{12}/l)$	7-13	5-10	9-15	8-18	5-8	6-9	5-10
PCV (l/l)	0.32-0.53	0.24-0.46	0.27-0.45	0.22-0.38	0.32-0.50	0.37-0.55	0.24-0.45
Hb (g/l)	110-190	80-150	90-150	80-120	100-160	120-180	80-150
MCV (fl)	37-58	40-60	28-40	16-25	50-68	65-75	35-50
MCH (pg)	12-20	11-17	8-12	5-8	17-21	22-25	12-17
MCHC (g/l)	310-380	300-360	310-340	300-360	300-340	300-340	290-340
Reticulocytes (10 ⁹ /l)						5-15	2-16
WBC number (10 ⁹ /l)	5.4-14.3	4.0-12.0	4.0-12.0	4.0-13.0	11.0-22.0	6.0-17.0	7.0-17.0
Neutrophils (10 ⁹ /l)	0-0.10	0-0.12	0-0.12	0-0.12	0-0.90	0-0.45	0-0.30
Lymphocytes (10 ⁹ /l)	1.5-7.7	2.5-7.5	2.0-9.0	2.0-9.0	4.3-13.6	1.0-3.6	1.0-6.0
Monocytes (10 ⁹ /l)	0-0.10	0.02-0.84	0-0.75	0-0.55	0.20-2.20	0-0.50	0-0.60
Eosinophils (10 ⁹ /l)	0-0.10	0-2.40	0-1.00	0.05-0.65	0.06-2.40	0-0.60	0-0.90
Basophils (10 ⁹ /l)	0-0.30	0-0.20	0-0.30	0-0.12	0-0.40	0-0.10	0-0.10
PLT number (10 ⁹ /l)	104-368	800-1,100	800-1,100	300-600	520	200-500	300-600

Appendix I. Hematological reference values according to SI units in domestic animals.

RBC: Red Blood Cell; PCV: Packed Cell Volume; Hb: Hemoglobin; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; WBC: White Blood Cell; PLT: Platelet.