Introduction into the Clinical Approach to Anemia – Clinical Signs & Diagnostics Including Blood Smear, Hematology Analyzers, & Other Clinical Tests

Introducere în abordarea clinică a anemiei – Semne clinice și diagnostice incluzând frotiu de sânge, analize hematologice, alte teste clinic

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Anemia is not a diagnosis in itself but is a common clinical sign and laboratory test abnormality in companion animals. Thus, anemia may indicate a specific erythrocyte problem or can be associated with other organ disorders. Because anemia and other hematological abnormalities occur so frequently, a complete blood cell count is generally requested in the diagnostic assessment of any seriously ill patient. Anemia is defined as a decrease in the red blood cell (RBC) mass as expressed by a reduction in number of circulating RBCs, hematocrit, and hemoglobin. Clinical signs of anemia result from decreased oxygen-carrying capacity, reduced blood volume, underlying disease, and the adjustments made to increase the efficiency of the erythron. The severity of clinical signs depends on the rapidity of onset, the degree and cause of anemia, and the extent of physical activity.

Anemias are differentiated into regenerative and non-regenerative anemias depending on the response of the bone marrow to anemia. Regenerative anemias are associated with hemolysis and blood loss. Non-regenerative anemias refer to reduced or ineffective erythropoiesis for the degree of anemia. All forms of anemia start of as being non-regenerative for the first few days until the bone marrow had time to react with the release of reticulocytes, but some remain non-regenerative. Hemolytic and blood loss anemias are often only mildly regenerative in cats. Anemias due to reduced or ineffective erythropoiesis develop generally slowly because of the long half-life of erythrocytes. Patients can adapt to the lower hematocrit, and therefore the clinical signs are often less severe and only noticed with severe anemia. Pallor is the typical clinical sign of non-regenerative anemias due to reduced or ineffective erythropoiesis often without any other helpful clinical features.

Erythropoietic response and diagnostic approach

The number of erythrocytes present in the circulation is a dynamic equilibrium between the production and delivery of erythrocytes into the blood circulation and their destruction or loss from circulation. The erythroid bone marrow response is mostly regulated by erythropoietin, a lineage-specific hematopoietic growth factor. Erythropoietin synthesis in the renal cortex is induced by anemia, although the actual sensor measures oxygen tension and recognizes only renal hypoxia. This hormone acts predominantly on erythroid precursor cells of the bone marrow known as burst-forming units-erythroid (BFU-E) and particularly colony-forming units-erythroid (CFU-E). At maximal stimulation, the bone marrow is capable of producing erythrocytes at 10-to 50-fold the normal rate. Erythropoietin also contributes to the maturation from the early committed erythroid precursors to fully hemoglobinized erythrocytes, which takes normally approximately 1 week. There is an inverse relationship between serum erythropoietin and hematocrit and only in anemia of chronic renal failure is there an absolute erythropoietin deficiency.

Reticulocyte counts

The most useful marker of accelerated erythropoiesis continues to be an increased number of reticulocytes in circulation. Because they contain residual RNA, which can be precipitated into a reticulum network and stained by certain supravital dyes such as new methylene blue or brilliant cresyl blue, reticulocytes can be readily enumerated. The latter stain is particularly suitable as it is relatively free of precipitates. On a vital stained blood smear, the number of reticulocytes is counted per 500 to 1000 erythrocytes and the reticulocyte result is reported as a percentage of cells examined (number per 100 cells). Few of the new in-house hematology instruments can also enumerate reticulocytes. Because canine reticulocytes contain strong aggregates, they are relatively easy to count. In contrast, cats produce two types of reticulocytes, namely aggregate and punctate reticulocytes. The aggregate reticulocytes correspond to the reticulocytes seen in dogs and indicate an active regenerative response.

The upper limit of a normal reticulocyte count is often stated as 1%; however, healthy small animals generally have <0.6 per cent reticulocytes per 100 erythrocytes. This number is not only increased by enhanced hematopoiesis, but also affected by anemia, as it depends on the number of circulating erythrocytes, and the duration of reticulocyte maturation in circulation. Therefore, various adjustments to the reticulocyte count are being used. If the erythrocyte count is available, the absolute reticulocyte count can be calculated and is less than 60,000/µL in a healthy animal:

$$\frac{\%\ \text{reticulocytes} \times \text{RBC}/\mu\text{L}}{100} = \text{reticulocytes}/\mu\text{L}$$

The absolute reticulocyte count has now been adopted as the preferred expression of erythroid regeneration. Several automated hematology analyzers have incorporated staining to detect reticulocytes and provide the absolute reticulocyte count; it should be noted that some automated counts may be falsely increased in the presence of Heinz's bodies and Howell-Jolly bodies as well as punctate reticulocytes (cats). However, if the absolute erythrocyte count is not known, the correction for anemia can also be made on the basis of the patient's hematocrit compared with the average normal packed cell volume (PCV) value (dogs 45%; cats 37%) and is normally less than 0.4%.

$$\%\ \text{reticulocytes} \times \frac{\text{patient's PCV}}{\text{normal PCV}\ (\text{dogs } 45;\ \text{cats } 37)} = \begin{matrix}\text{corrected} \\ \%\ \text{reticulocytes}\end{matrix}$$

A low absolute reticulocyte count or corrected reticulocyte percentage provides the best evidence for a lack of a bone marrow response. Although not as accurate, other hematologic manifestations may be used to indicate increased erythropoiesis, when a reticulocyte count is not available. On a regularly (Wright; Diff-Quik) stained blood smear, polychromatophilic cells represent erythrocytes recently released from the bone marrow and are equal to reticulocytes. As their blue-grey tint color is due to the presence of RNA, their numbers correlate well with the reticulocyte count. When more than one polychromatophilic cell is recognized per microscopic oil immersion field, accelerated erythropoiesis is suggested. Polychromatophilic erythrocytes are often macrocytic, which indicates that these cells are released prematurely from the marrow, thereby contributing greatly to the high mean cell volume (MCV) and red cell distribution width (RDW) as well as anisocytosis of regenerative anemias and low MCHC (still need to be hemoglobinized). It should be noted that a macrocytic anemia is not always regenerative, but may indicate a maturation problem such as myelodysplasia, feline leukemia virus (FeLV) infection or folate deficiency in cats.

Normoblasts or nucleated RBCs

Nucleated erythrocytes also known as normoblasts or metarubrocytes with variably shrunken nuclei are rarely found in blood of healthy animals (less than 1 per 100 white blood cells). Depending on the hematology instrument they may be not counted or under the white blood cells (WIC vs WOC). Large numbers may accompany a marked regenerative response in anemic patients. However, nucleated RBCs may also be seen in patients without reticulocytosis and regenerative response because of a breakdown of the barrier between marrow and vasculature. In fact, the highest numbers of normoblasts are observed in acute lead poisoning in the absence of anemia. Mild to moderate normoblastosis with nonregenerative anemia may be seen with myeloproliferative disorders, dyshematopoiesis, extramedullary hematopoiesis, hemangiosarcoma, and sepsis. Thus, normoblastosis should not be equated with a regenerative marrow response without confirmation by a reticulocyte count.

Bone marrow examination

If the anemia is characterized as non-regenerative on the basis of an absence of reticulocytosis, bone marrow examination is indicated. In non-regenerative anemia, the bone marrow cellularity can be decreased, normal or even increased. However, the ratio of myeloid to erythroid elements in the bone marrow is generally below one. Although the erythropoietin response is lineage specific, independent stimulation of thrombopoiesis or granulopoiesis or lack thereof may also be present. Bone marrow examination may provide helpful information about an underlying cause (cancer, infection) or marrow iron deposition in patients with non-regenerative anemia that would otherwise remain undetected. Bone marrow examination from humerus and even ribs has become more standard.

Degree of anemia

The degree of the anemia can be simply defined by the determination of a packed cell volume (PCV) by microcentrifugation or hematocrit as part of a complete blood cell count (CBC). The PCV or microhematocrit is directly measured, whereas the hematocrit is calculated from the mean cell volume times the erythrocyte number. Typically, these two values should be equal, although in vitro crenation, swelling, or lysis of erythrocytes and inadequate sedimentation by the microcentrifuge could cause erroneous results. An EDTA-anticoagulated sample is ideal for most hematologic tests. It is important to assure prompt mixing of blood with anticoagulant in the correct proportion; therefore, for small sample sizes, pediatric or microtubes should be used.

Blood hemoglobin values are generally used in human medicine rather than a PCV or hematocrit to define the degree of the anemia. The point-of-care HemoCue and other instruments have been validated and can be applied in any species to assess blood hemoglobin concentrations, whereas hemoglobin measurements by some of the laboratory CBC instruments may be affected by lipemia and leukocytosis. There is a good correlation between PCV and hemoglobin concentration in that the PCV is roughly three times the hemoglobin. The HemoCue has proven invaluable when using Oxyglobin as a transient oxygen carrier in the treatment of anemia because it allows for accurate total blood and plasma hemoglobin determinations in practice. The hemoglobin in erythrocytes (times 3 will give the PCV) is obtained by subtracting the plasma hemoglobin from the blood hemoglobin. Mild degrees of hemoglobinemia may also be caused by intravascular hemolysis or could be blood collection or storage artifacts; a second blood sample and urinalysis including examination of the urine sediment will discover the difference.

Testing for anemias

One of the most important but simple blood tests for an anemic patient is the microscopic evaluation of a fresh EDTA blood sample. For instance, the presence of polychromasia and anisocytosis on a blood smear suggests a regenerative anemia. The finding of various morphologic erythrocyte abnormalities may determine a cause of the anemia, such as hypochromasia with iron deficiency anemia or agglutination, and spherocytosis with immune-mediated hemolytic anemia, schistocytes with disseminated intravascular coagulation, Heinz bodies with oxidative insults, and erythrocyte parasites seen with infectious anemias. Note that parasitemias are often transient and quickly disappear after treatment.

Current standards in small animal medicine also demand a CBC by a hematology instrument in order to better characterize the anemia and other associated hematologic abnormalities. Each of these hematology tools has to be validated for small animals, as instruments in human laboratories are not set to accurately assess canine and feline blood cells. The small size of feline red blood cells and large size of feline platelets present a particular challenge. Impedance instruments were the first ones to provide more details regarding red cell indices and white blood cell (WBC) differentials. However, these instruments continue to have problems in counting and differentiating accurately blood cells; for instance, nucleated red blood cells may not be separately assessed. Furthermore, some instruments may perform well with blood from healthy animals but are inaccurate when abnormalities are encountered. In the past, validated instruments for CBCs were only available in large reference laboratories and teaching hospitals because of the expense and expertise required to run the instrument.

Several smaller hematology instruments have become available for use in practice, which can now provide immediate and in some cases similarly accurate results as the large laboratory instruments. The QBC was the first of its kind attempting to further differentiate the buffy coat to provide some estimation of a WBC differential and reticulocytes but was, unfortunately, inaccurate. The most recent advances in laser technology utilized in novel in-practice hematology instruments appear particularly promising in providing small animal clinicians not only accurate red cell parameters, including reticulocytes, but also better WBC differentials and platelet counts and cell volumes. Although these instruments can be used in practice, they will not replace the clinical pathologist who can provide a careful and better review of all hematologic data ill patience, including the cytologic evaluation of blood smears and bone marrow aspirates.

Bone Marrow Evaluation

In the case of nonregenerative anemias, bone marrow evaluation often becomes crucial. Although overall cellularity and the presence of megakaryocytes can be readily determined, the various cell lineages and degree of maturation in the bone marrow is left to the experienced eye. Similarly, special stains for the presence of iron stores (healthy cats generally do not have stainable iron in their marrow) and immunohistochemistry for differentiation of the various hematopoietic precursor cells, as well as the recent application of flow cytometry of blood or marrow cells, is only done in reference clinical pathology laboratories.

Iron, Vitamins, Erythropietin

In addition to this general assessment of anemia, several other tests have become available for the clinical characterization of anemias. Instead of simply determining serum iron, there are now opportunities to define the iron saturation and serum ferritin. Serum iron and ferritin concentrations are expected to be low in iron deficiency; ferritin is an acute-phase protein and therefore its concentrations vary significantly in small animal patients and are

increased with inflammation. Furthermore, as cobalamin (vitamin B_{12}) and folate deficiencies contribute to nutritional anemias, serum cobalamin and folate concentrations may need to be measured in cases of nonregenerative megaloblastic anemias. Acquired and inherited forms of cobalamin malabsorption have been recognized. Serum erythropoietin assays have been validated to discover relative and absolute erythropoietin deficiencies in non- or poorly regenerative anemias, and absolute and relative erythropoietin deficiencies have been documented with chronic renal failure and cancer, respectively, leading to anemia. It should be noted that antierythropoietin assays for pure red cell aplasias following recombinant human erythropoietin therapy are not generally available.

Blood Chemistry and Toxicology

Along with the CBC, a serum chemistry screen is often requested to suggest renal and hepatic failures or endocrinopathies, but it may also reveal a secondary hypophosphatemia responsible for intravascular hemolysis. Toxicology laboratories now offer identification of a large variety of substances including heavy metals such as zinc, lead, and copper, causing various forms of anemias and anticoagulant rodenticides, as well as drug levels in blood. Many drugs have been implicated to cause various forms of anemia and other cytopenias by immune and bone marrow suppressive processes.

Hemostatic Tests

Standard hemostatic evaluation includes determination of platelet count and morphology, plasma von Willebrand factor, and coagulation parameters. Simple in-practice tests to assess hemostasis include platelet estimates on blood smears, a buccal mucosal bleeding time, and an activated clotting time. Microscopically, >15 platelets per high-power oil emersion field are normally found, and macroplatelets may suggest regeneration or myelodysplasia. In case of reduced platelet numbers, aggregates may be detected on the margins of the blood smear, as particularly feline platelets are readily activated, which leads to pseudothrombocytopenia. The buccal mucosal bleeding time (normal <3.5 minutes) is performed only if platelet numbers are adequate and drugs known to interfere with platelet functions have been excluded. The buccal mucosal bleeding time assesses exclusively primary hemostasis and is prolonged with hereditary and acquired thrombopathias and von Willebrand's disease. The tube activated coagulation time is a simple and inexpensive way to evaluate overall coagulation (secondary hemostasis) as it is expected to be prolonged with any coagulopathy, except isolated factor VII deficiency (seen in beagles).

The classic coagulation tests, partial thromboplastin time and prothrombin time (PT), were only offered in clinical pathology laboratories on fresh citrated plasma, which had to be immediately separated from red cells and shipped frozen or on ice for analysis. However, partial thromboplastin time and PT can now also be done with a simple point-of-care instrument on fresh citrated whole blood in practice allowing for immediate identification of any coagulopathy. The protein induced by vitamin K antagonism or absence (PIVKA-) test is a PT offering no advantage over the regularly performed PT and a prolongation is not specific for rodenticide poisoning. The PIVKA test has long been replaced in human medicine by the standardized PT. Special coagulation tests are available to determine a specific factor deficiency at Cornell's Comparative Hemostasis Laboratory and PCR-based tests are also available for von Willebrand's disease in a few breeds including Doberman pinschers, Scotties, Shelties, Kookier, and German short and wirehair pointers through commercial laboratories. However, von Willebrand's disease, a primary hemostatic disorder, is best diagnosed with a plasma von Willebrand factor analysis (ELISA test utilizing citrated or EDTA anticoagulated blood).

Immunological Assays

A diagnosis of immune-mediated hemolytic anemia requires the identification of persistent autoagglutination after saline washing (3 times with physiological saline), marked spherocytosis, and a positive direct Coombs' test (antiglobulin test), whereas agglutination on the slide and response to immunosuppressive therapy are insufficient evidence for an immune process. True autoagglutination that does not break up after saline washing precludes the performance of both Coombs' test and blood typing as the endpoints of these tests is agglutination. Coombs' tests have to be done with species-specific reagents and proper techniques in special laboratories in order to minimize false-positive and false-negative results. Dogs with active immune-mediated hemolytic anemia (IMHA) should have a positive Coombs' test result, even when treated for a couple of days with steroids. Whereas dogs often have a primary/idiopathic form of IMHA, cats rarely have IMHA and the hemolytic anemia is generally due to an underlying disease or trigger. Thus, even when documenting an immune destruction of erythrocytes, it is important to rule out other underlying disease processes as cause. Similarly, platelet-associated antibody tests are offered on a limited basis for the confirmation of immune-mediated thrombocytopenia, due to the small number of platelets flow cytometric methods appear preferable.

Furthermore, as anemic patients may be in need of a transfusion, each patient should also be blood typed, and if previously transfused, cross-matched to assure the administration of safe and effective transfusions. There are simple in-practice kits available to type dogs for DEA 1.1 and cats for type A, type B, and type AB. There are also protocols available for performing a cross-match in practice to assure compatible blood transfusions.

Infectious Disease Screening

Although microscopic and serologic screening tests for infectious and parasitic diseases have been available for many years, the diagnosis of many blood-borne diseases remains challenging. For instance, there is no serologic test for hemobartonellosis and serologic tests for babesiosis are cross-reacting between species of *Babesia*. However, the recent introduction of polymerase chain reaction (PCR)-based methods allows a more sensitive and precise diagnosis of different forms of infectious diseases such as babesiosis, including *Babesia canis* subtypes and *Babesia gibsoni*. PCR tests can also distinguish between feline hemobartonellosis caused by *Mycoplasma haemofelis* or less likely *M. haemominutum*. Serology and PCR tests are also available for aiding in the diagnosis of ehrlichiosis and leishmaniasis. In addition, infections with *Leptospira* spp have reemerged as an important cause of acute systemic illness and hemolysis. Simple SNAP tests are available to determine exposure to *Ehrlichia canis*, *Bartonella burgdorferi*, *Anaplasma sp.* and *Dirofilaria immitis*.

Genetic Disease Testing

Finally, several hereditary erythrocyte disorders have been discovered that may mimic acquired hemolytic anemias such as IMHA, and biochemical and molecular genetic tests for some of these disorders have become available (www.vet.upenn.edu/penngen). In particular, PCR tests are offered for phosphofructokinase deficiency in English springer and few other breeds (albeit this can also occur in mixed breed dogs). PFK deficiency can cause severe intermittent anemia and myopathy. PCR testing is also available for pyruvate kinase (PK) deficiency in basenjis, beagles, West Highland white and cairn terriers, dachshunds, and Abyssinian and Somali cats. Interestingly, PK deficiency in any canine breed is associated with severe chronic anemia and osteosclerosis, whereas this enzyme deficiency in cats results in an intermittent anemia without osteosclerosis. Similarly, PCR tests are available for canine leukocyte adhesion deficiency in Irish setters and red and white setters, a disorder associated with a massive leukocytosis and anemia. With respect to hereditary hemostatic defects, several PCR-based tests (besides the ones for mutations in the von Willebrand gene) have

emerged, including coagulopathies FVII, VIII, IX XI and XII, macroplateletes, and Glanzman thrombasthenia.

In conclusion, the diagnostic approach to anemias in small animals has been assisted greatly by new tools and tests. Their appropriate use will permit a more precise and rapid diagnosis and thereby effective therapy.

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