BEYOND THE NUMBERS: CYTOLOGIC EVALUATION OF PERIPHERAL BLOOD SMEARS

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Recent advances in the field of bench top/point-of-care hematology analyzers have greatly improved both acquisition and analysis of peripheral blood in animals. However, limitations still exist in the ability of automated analyzers to appropriately identify many cell types. Furthermore, no analytic analyzer is capable of detecting and identifying Hemoparasites, nucleated erythrocytes or morphologic changes. Therefore, visual examination of well-prepared blood smears in addition to the complete blood count (CBC) remains critical to appropriate interpretation of veterinary diagnostic hematology.

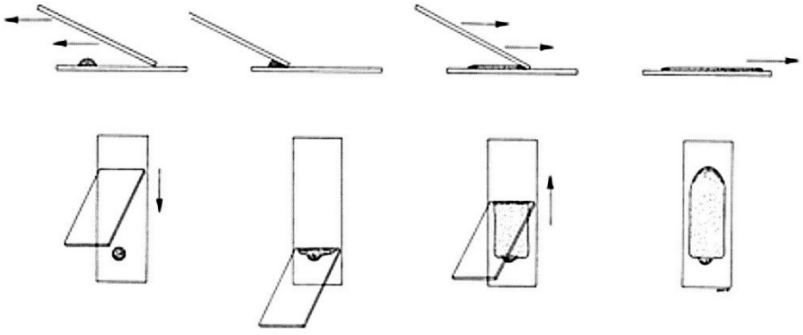
A relatively recent survey of veterinary practices found that, although >90% of respondents owned "in-house" laboratory equipment, less than 1/3 performed routine blood evaluation if the sample was flagged with a problem. Even fewer performed a manual backup of the WBC differential counts all or most of the time[1]. Oversight of blood smear examination in clinical practices is likely multifactorial. Most practices rely on technical staff to perform in-house procedures including hematology and many assistants have voiced that they do not feel adequately trained to perform manual procedures while others cite a lack of time to create and review blood smears[2]. These limitations exist in light of assistants' clear desire to provide high quality and accurate results for patients.

This brief review will cover the basics of how to efficiently create a good quality blood smear, evaluate the smear and recognize some clinically significant features. Case-based examples are provided for clarification of why blood smear examination should be routinely performed in conjunction with a CBC, particularly when patients are clinically ill and/or if numeric abnormalities are detected.

Making a quality blood smear: A blood smear should be made on a new, previously unused, clean glass slide. Never reuse slides. Smears may be made from whole blood without anticoagulant or acquired from and EDTA or heparin tube (note that heparin alters morphologic characteristics of cells and is reserved mainly for sampled obtained from avian and reptile/amphibian species). Also, avoid exposure to formalin as even the fumes can negatively affect the staining of the sample.

Protocol:

1. Place a single drop of blood near the edge of the slide
2. Using another slide placed at a 45° angle, draw it back until it meets the drop of blood
3. Allow the blood to spread along the edge of the angled slide
4. Gently push the angled slide toward the opposite edge while evenly applying gentle pressure



Optimally the smear should cover approximately 2/3 of the slide and contain a well-spread monolayer and a feathered edge. Based upon the density of the cells and viscosity of the sample (e.g. due to hyperglobulinemia), the angle of the spreader slide may need to be altered to obtain a good smear.

Staining the slide: Allow the slide to fully air dry. **NEVER** "heat fix" the sample with a lighter. Heat fixation is useful for prepping bacterial cell walls for gram staining, but it should never be done for cytologic assessment. Exposure to open flame causes marked cell damage and often alters staining. Staining the slide before it is completely dry also causes artifacts such as vacuoles in the cells and crenation.

Most clinics use a quick stain procedure such as Diff-Quick®. These staining kits are relatively inexpensive, easy to use and provide an excellent option to automated staining methods used in larger labs. However, they are fallible and understaining, overstaining and contamination of the slides are often noted in submitted, pre-stained slides. These errors can usually be avoided by changing out the stain regularly and performing a consistent, even staining protocol. We usually recommend 5-7 full dips in each jar, blotting the runoff before moving on to the subsequent jar, then rinsing with cool tap water and again allowing it to air dry.

Protocol Summary:

1. 5-7 full dips in jar 1 (contains an alcohol fixative)
2. Blot edge of slide on a paper towel
3. 5-7 full dips in jar 2 – Solution I (eosinophilic stain)
4. Blot edge of slide on a paper towel
5. 5-7 full dips in jar 3 – Solution II (basophilic stain)
6. Gently rinse with tap water
7. Place upright to drain and air dry

Slide assessment: Begin at low power (10x objective). Perform a full assessment of the smear paying particular attention to overall erythrocyte numbers, distribution of the cells and noting any large atypical cells or structures. Large platelet clumps, microfilaria and abnormal erythrocyte aggregation (e.g. agglutination or rouleaux formation) are easiest to identify at lower power. Additionally, large atypical (e.g. neoplastic) cells are often easiest to first identify at low power. Scan the feathered edge of the slide at 10x objective for additional platelet clumps and possible parasites as larger structures are often dragged to this location during spreading of the cells. Once the initial scan is completed, move to the monolayer and switch to 40x or 50x objective to perform a 100-200 leukocyte differential count. Cells are counted with a manual or electronic counter and reported as a percentage (%). The actual numeric count of each leukocyte category is then calculated from the automated count (e.g. % neutrophils x WBC count = absolute neutrophil count).

Nucleated red blood cells (nRBCs) are not detected by automated counters, thus experience and attention is needed to recognize nRBCs and they are NOT counted as part of the leukocyte differential. nRBCs are counted separately and some automated differential counters have a separate key to quantitate them independent of the 100-cell leukocyte differential count. If more than 5 nRBCs are identified per 100 WBCs, then the total leukocyte count should be corrected via the following formula:

Corrected WBC count = Automated WBC count X 100
 # nRBCs + 100

Assess any leukocyte abnormalities such as toxic changes, abnormal or unknown cell populations, presence of leukocyte inclusions or other abnormal structures. Toxicity, in particular, can be subtle but marked toxic changes are highly prognostic, thus identification is critical[3,4]. Examine and report any erythrocyte abnormalities including presence of anisocytosis, polychromasia, types of poikilocytes, and/or presence of organisms or atypical inclusions.

Finally, assess platelets. Do platelets appear adequate, increased or decreased? Are large/giant platelets present? As necessary, move to high power, 100x objective, for further identification of cell features and abnormalities. Platelet estimates are typically performed at 100x oil objective and each platelet recognized represents approximately 15,000 – 20,000 platelets/μL. Thus, optimally, patients should have between 8 – 10 platelets per 100x field unless clumps are present.

Examples of toxic changes[5] and erythrocyte morphologic abnormalities (poikilocytes) are provided in addendums 1 and 2 (respectively)[6].

While uncommon, microorganisms can be found in peripheral blood. Bacteremia is rare but other organisms can be detected on careful examination of blood smears. Erythroparasites include organisms such as *Mycoplasma haemofelis*, *Cytauxzoon felis* and *Babesia spp*. Parasites found in leukocytes include rickettsial organisms (e.g. *Ehrlichia spp.*, *Anaplasma phagocytophilum*), protozoa (e.g. *Hepatozoon spp.*) and fungal organisms. Additionally, viral inclusions can identified in acute distemper infections in puppies. Many infectious diseases have regional distributions; therefore, travel history including adoption information are very helpful to indicate exposure.

Protocol Summary:

1. Scan the slide at low (10x) objective
    a. Note erythrocyte density
    b. Note erythrocyte distribution
        i. Agglutination present?
        ii. Rouleaux present?
    c. Assess for large platelet clumps
    d. Assess feathered edge for abnormal cells, structures or organisms
    e. Assess any background abnormalities: e.g. basophilic staining plasma or lipid
2. Move to 40x or 50x objective:
    a. Assess erythrocyte morphology
        i. Assess any poikilocytes trends (rare changes are not reported)
        ii. Assess any anisocytosis and polychromasia (particularly if anemic)
        iii. Note presence of nRBCs (see next)
    b. Assess leukocyte morphology
        i. Perform leukocyte differential count
        ii. If >5 nRBCs per 100 WBCS, report and correct the WBC count
        iii. Note any toxic changes
        iv. Note small platelet clumps if present

3. As necessary, move to high power (100x oil) objective:
    a. Scan for platelet numbers; estimate if low
    b. Assess if large/giant/macroplatelets are present
    c. Assess if organisms are suspected in RBCs or WBCs

Minimizing error: Errors in reporting of laboratory data, including blood smear assessment, are common[7]. In human laboratory studies, up to 75% of errors occur before the sample is analyzed, for example during collection, preparation or processing. These are referred to as "preanalytic errors" are the easiest to remedy with careful preparation and attention to detail. Analytical errors are those errors resulting from poor test precision and inappropriate interpretation due to inexperience. These may be more difficult to initially detect and or remedy as they may be due to analyzer performance or a lack of technical training. Analytic errors are best avoided by regular quality control (QC) and assessment (QA). This can be accomplished by routinely running control samples and adequately training personnel. Post analytic errors are those that occur after the sample has been processed and results obtained. These may include misinterpretation of results or mistakes in transcribing data. Again, attention to detail and care in ascertaining correct patient identification and application of findings are necessary to avoid this latter error types.

Summary: Examination of the blood smear is an integral part of peripheral blood assessment. Many abnormalities may be unfamiliar to the individual examining the smear. The point of examination is not to recognize every and all abnormality present, but to note consistent abnormal trends. Are there many cells that you cannot recognize? Are there abnormal shapes and/or sizes of erythrocytes? Are there structures that you are unfamiliar with? As most blood will have minimal, if any changes, an observer should be well acquainted with how peripheral blood SHOULD look, thus, if a sample appears abnormal, it probably is! Finally, human error is inevitable and mistakes will occur; however, with attention to detail, education and knowing when and who to ask for help, diagnosis can often be achieved and patient care appropriately addressed.

Case reports:

"ZAMBA": A 9-year-old, female-spayed DSH cat was presented to her veterinarian for recent onset of decreased appetite and occasional vomiting. The veterinarian acquired both hematologic and clinical biochemistry panels via in-house bench top analyzers. No significant abnormalities were identified on the CBC report. The veterinarian created a smear of the blood and noted cells of questionable identity. Therefore, an additional slide was prepared and both the pre-stained and unstained slides were submitted to Oregon State's Veterinary Diagnostic Laboratory (OSU-VDL) for review. One slide was stained with Modified Wright's stain and both slides were examined with similar findings noted on both. Cell counts correlated with the automated analyzer results reported by the referring DVM. A leukocyte differential count was performed which revealed the following: 66% neutrophils, 11% monocytes, 10% lymphocytes, 9% mast cells, 3% eosinophils, and 1% basophils. Therefore, presence of atypical cells in circulation were identified as mast cells. Based upon this finding additional diagnostic workup was pursued. As suspected, splenic mastocytosis was found in this cat.

<u>Interpretation</u>: Mastocythemia (circulating mast cells)

Circulating mast cells in cats are most likely to represent mast cell neoplasia in cats[8]. These patients typically have visceral, usually affecting the spleen, mast cell tumors. Mast cell neoplasia accounts for 15–26% of splenic disease in cats. Buffy coat analysis has also been shown to be very useful for identifying mast cells in circulation of patients with systemic mast cell neoplasia.

"HONEY BEAR": A 10-year-old FS dog was referred for further diagnostic work up for a marked anemia and lymphocytosis. At presentation the dog was recumbent with pale mucous membranes. Prior automated CBC had been obtained via a point-of-care in-house analyzer with the following results:

Parameter	Patient Value	Flag	Reference Interval	Units
PCV	8.5	↓	37 – 54	%
Hgb	3.6	↓	13 – 19	g/dL
MCV	69.7		64 – 74	fL
MCHC	42.4	↑	34 – 36	g/dL
WBC	44.7	↑	6 – 17	$x10^3/\mu L$
Neutrophils	20.6	↑	3 – 11.5	$x10^3/\mu L$
Lymphocytes	20.1	↑	1 – 4.8	$x10^3/\mu L$
Monocytes	4.02	↑	0.15 – 1.35	$x10^3/\mu L$
Platelets	154	↓	200 – 600	$x10^3/\mu L$

The analyzer software provided possible differentials for the marked lymphocytosis at the bottom of the report which included a primary consideration of lymphoid leukemia. Thus, repeat CBC and blood smear evaluation were performed. Examination of the blood smear revealed numerous nucleated red blood cells (nRBCs) with approximately 42 counted per 100 leukocytes during a differential count. Additionally, large numbers of spherocytes and polychromatic RBCs were identified. Approximately 20% of the leukocytes consistent of band neutrophils with moderate toxic change. Multiple small platelet clumps were present on the smear. The leukocyte count and differential were corrected for the of presence of nRBCs:

Parameter	Patient Value	Flag	Reference Interval	Units
PCV	8.5	↓	37 – 54	%
Hgb	3.6	↓	13 – 19	g/dL
MCV	69.7		64 – 74	fL
MCHC	42.4	↑	34 – 36	g/dL
WBC	31.5	↑	6 – 17	$x10^3/\mu L$
Neutrophils	21.7	↑	3 – 11.5	$x10^3/\mu L$
Bands	6.3	↑	< 0.3	$x10^3/\mu L$
Lymphocytes	0.9	↓	1 – 4.8	$x10^3/\mu L$
Monocytes	2.5	↑	0.15 – 1.35	$x10^3/\mu L$
Platelets	Adequate		200 – 600	$x10^3/\mu L$

Interpretation: Immune-mediated hemolytic anemia (IMHA) with an inflammatory leukogram

Artifactual "hyperchromia" is due to hemolysis and an inflammatory leukogram is common with IMHA. Evidence of regeneration and spherocytes are also consistent with immune-mediated RBC destruction and platelet clumping may indicate hypercoagulability. The dog was given a whole blood transfusion

and immunosuppressive therapy was initiated. No evidence of underlying neoplasia/leukemia was detected. Nucleated RBCs are counted as lymphocytes by automated analyzers which necessitates manual leukocyte differential counting[6]. Additionally, automated analyzers are unable to differentiate leukocyte abnormalities such as band neutrophils and toxic changes[5].

"BONGO": The sample was from a 1.5-year-old, male-castrated small-breed dog with chronic weight loos and intermittent diarrhea. Whole blood and prior CBC report, was submitted to the Oregon State Clinical Pathology Lab for examination. Notable abnormalities included marked anemia (HCT 13.1%; RR = 37 – 55) and a leukocytosis with a left shift neutrophilia and increased numbers of eosinophils. Additionally, marked thrombocytosis was reported at 1,534 $x10^3/\mu L$ (RR: 200 – 500 $x10^3/\mu L$) On evaluation of the smear, anemia was confirmed and moderate anisocytosis was present. Occasional polychromatic erythrocytes and frequent hypochromic erythrocytes, microcytes and spherocytes were also identified. A leukocytosis with a predominance of poorly preserved neutrophils was confirmed cytologically and numerous abnormal intra- and extracellular fungal yeast were identified throughout. Many structures exhibited budding. Only rare eosinophils were found and platelets appeared to be within normal parameters.

Interpretation: Disseminated histoplasmosis

Histoplasma capsulatum is a dimorphic fungus found in soil in many parts of the world[9]. In the U.S. most cases occur in the Ohio and Mississippi river valleys. Infection is by inhalation of spores which disseminate to other organs. Infected dogs usually present with chronic gastrointestinal signs, typically colitis. Anemia is common and likely multifactorial from poor iron absorption and immune-destruction (spherocytosis). Budding is less commonly seen in tissues and, when present, is often due to immune-suppression. As the organism is less commonly encountered in other parts of the U.S., infections are often overlooked and clinical signs misdiagnosed as immune-mediated disease (e.g. inflammatory bowel disease) or neoplasia; therefore, inappropriate treatment with glucocorticoids is contraindicated and can worsen clinical disease. Automated platelet and eosinophil counts in this sample were likely due to presence of intra- and extracellular organisms.

"MARCO": A 9-year-old male castrated DSH cat was presented to the teaching hospital with a history of lethargy, poor appetite and weight loss. Upon physical examination organomegaly was identified. The patient was referred to the veterinary teaching hospital for further workup for suspected neoplasia. A CBC was performed which revealed a mild-moderate microcytic normochromic nonregenerative anemia and a mild inflammatory leukogram. On examination of a blood smear marked poikilocytosis including numerous keratocytes and blister cells and frequent schistocytes was identified. Occasional acanthocytes were also found. These findings are consistent with erythrocyte destruction due to abnormal vascular channels ("microangiopathy"). Fine-needle aspirates of the spleen and mesenteric lymph nodes were obtained and lymphoma was diagnosed in both tissues.

Interpretation: Marked microangiopathic destruction of red blood cells

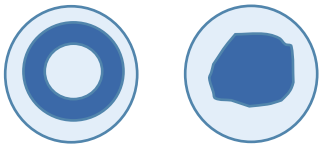
Keratocytes and blister cells are typically associated with intravascular trauma to erythrocytes[6]. This mechanism is known as microangiopathic hemolytic anemia (MHA) and is due to blood flow through abnormally narrowed or tortuous blood vessels; often the spleen, liver or bone marrow. MHA may therefore occur secondary to several conditions including neoplasia, bone marrow disease, renal

disease, and increased fibrin deposition due to massive inflammation or disseminated intravascular coagulation (DIC). RBCs flowing through these narrowed vessels are sheared and evidence of hemolysis may be noted. In cats, liver disease has also been associated with production of blister cells and schistocytes due to both microangiopathy and oxidative membrane damage[10]. Severe iron deficiency anemia can also cause keratocyte and blister cell production as cells become more fragile and less deformable due to iron loss[11]. This may be seen in cases of chronic gastrointestinal disease including IBD and infiltrative neoplasia. Although GI involvement of lymphoma was not investigated in this case, and iron levels were not measured, iron deficiency may have been a contributing cause of the massive erythrocyte destruction seen in Marco's blood.

Addendum 1: Toxic changes

Toxic changes include abnormalities of both the cytoplasm and nucleus of affected neutrophils. These are caused by accelerated production in the bone marrow and, thus, are associated with inflammation and consumption of normal, mature cells and increased demand. Neutrophil toxic change is often an early indicator of acute systemic illness and has been associated with disease severity, case fatality, hospitalization duration, and cost of treatment in dogs and cats[6,7].

Toxic change may accompany a left shift; however, they may also occur independently of any numerical leukocyte abnormalities. Although they are common with infectious diseases, they can also be found in noninfectious causes of inflammation such as pancreatitis, necrotizing tumors, bone marrow disease, toxin exposure, etc.

Cytoplasmic basophilia	retention of ribosomes and endoplasmic reticulum (ER)	
Döhle bodies	aggregations of rough ER in the cytoplasm (common in cats and horses); appear as blue smudges which may be mistaken as organisms	
Vacuolization	lysosomal activity; vacuoles may be large or small, giving the cells a "foamy" appearance	
Primary granules	pink cytoplasmic granules which are retained mucopolysaccharides	
Abnormal nuclei	hyposegmented cells; "donut" nuclei	

Addendum 2: Poikilocytes

Poikilocytosis is a generic term indicating a change in erythrocyte shape. As it is a nonspecific, the type of shape change is far more useful for interpretation and establishment of differential diagnoses. Common poikilocytes found in dog and cat blood are briefly described below.

Acanthocytes or "spur cells": RBCs with blunt or club-shaped spicules of different lengths projecting from their surface at irregular intervals. They may occur secondary to fragmentation injury, in friable cells due to iron deficiency anemia, or secondary to abnormal lipids (e.g. liver dysfunction).

Echinocytes are speculated/crenated RBCs. Projections of the cell membrane may be sharp or blunt and tend to be evenly spaced around the circumference. They are generally caused by altered osmolality in plasma and, thus commonly found in dehydrated patients or those with electrolyte depletion. They are also commonly seen in association with snake bites as the venom alters the cell membrane integrity. They also occur as artifacts due to prolonged storage in EDTA or exposure to moisture.

Heinz bodies: these are small aggregates of hemoglobin which create a protrusion on membranes. They resemble a small button or, in some planes, appear as a small clear space in the RBC. They indicate oxidative damage to the erythrocytes. Few Heinz bodies are common in blood of cats; however, large numbers are indicative of severe oxidative injury in all species and blood transfusion is indicated as the cells are unable to carry oxygen. They are related to toxins such as onions, garlic, zinc or skunk spray.

Howell-Jolly bodies: Nuclear remnants. May indicate regeneration, abnormal spleen or bone marrow function

Keratocytes or "blister cells" or "helmet cells": erythrocytes with small vesicles which may rupture, leaving a "bite-shaped" defect in the cell with projections. They are typically associated with fragmentation injury and may be found with acanthocytes and/or schistocytes. They have also been found in cats with Heinz bodies due to oxidant injury.

Schistocytes are red blood cell fragments. They usually reflect mechanical injury to erythrocytes. They may also be present in animals with severe iron deficiency anemia as cells become "weak" and prone to destruction due to hypochromia.

Spherocytes are dense round erythrocytes and smaller than normal RBCs. They lack central pallor and, thus, are difficult to definitively identify in species other than dogs. They result from removal of membrane-bound immune complexes by macrophages as RBCs circulate through the spleen. Post removal of a portion of the cell's membrane, the membrane reanneals creating a smaller, denser cell. Identification of more than few spherocytes in conjunction with anemia, indicates an immune-mediated component to the anemia.

Target cells: Erythocytes with a central region of hemoglobin surrounded by a clear rim of cytoplasm giving them a "bulls-eye" appearance. Few target cells are a nonspecific finding associated with many diseases in dogs. Large numbers of target cells, however, are most commonly found in patients with severe liver disease and abnormal cholesterol and phospholipid metabolism. They are rare in cats.

References:

1. Bell R, Harr K, Rishniw M, Pion P. Survey of point-of-care instrumentation, analysis and quality assurance in veterinary practice. *Vet Clin Pathol* 2015; 43(2):185-92.

2. Flatland B, Weiser G. The Elephant in the Room (and How to Lead It Out): In-Clinic Laboratory Quality Challenges. *J Am Anim Hosp Assoc* 2014; 50(6):375-82.

3. Segev G, Klement E, Aroch I. Toxic neutrophils in cats: Clinical and clinicopathologic features, and disease prevalence and outcome – retrospective case control study. *J Vet Intern Med* 2006; 20(1):20–31.

4. Aroch I, Klement E, Segev G. Clinical, biochemical, and hematological characteristics, disease prevalence, and prognosis of dogs presenting with neutrophil cytoplasmic toxicity. *J Vet Intern Med*. 2005; 19(1):64-73.

5. Weiser G. Interpretation of Leukocyte Responses in Disease. In: Veterinary Hematology and Clinical Chemistry 2nd ed. Wiley-Blackwell, Ames, Iowa. 2012; pp. 127-139.

6. Thrall MA. Erythrocyte Morphology. In: Veterinary Hematology and Clinical Chemistry 2nd ed. Wiley-Blackwell, Ames, Iowa. 2012; pp 61 – 74.

7. Vap LM, Harr KE, Arnold JE, et al. ASVCP quality assurance guidelines: control of preanalytical and analytical factors for hematology for mammalian and nonmammalian species, hemostasis, and crossmatching in veterinary laboratories. *Vet Clin Pathol*. 2012; 41(1):8-17.

8. Piviani M, Walton RM, Patel RT. Significance of mastocytemia in cats. *Vet Clin Pathol* 2013; 42(1):4-10.

9. Lin Blache J, Ryan K, Arceneaux K. Histoplasmosis. *Compend Contin Educ Vet*. 2011; 33(3):E1-10.

10. Christopher MM, Lee SE. Red cell morphologic alterations in cats with hepatic disease. Vet Clin Pathol. 1994; 23(1):7-12.

11. Fulton R, Weiser MG, Freshman JL, et al. Electronic and morphologic characterization of erythrocytes of an adult cat with iron deficiency anemia. Vet Pathol. 1988; 25:521-23.