BLOOD SMEAR BASICS

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Introduction

Although tremendous advances have been made in the field of point-of-care hematology analyzers, examination of a well prepared, well stained blood smear remains the cornerstone of veterinary diagnostic hematology. Even the most sophisticated hematology instruments are unable to consistently provide accurate differential cell counts, and no analyzer is capable of accurately identifying morphology changes, hemoparasites, neoplastic cells, etc. This review will cover the basics of how to approach blood smear evaluation in a consistent and systematic manner and will focus on recognition of clinically significant findings.

Making a quality blood smear

Although there are several techniques described for making blood smears, most people use the wedge or push technique.

- Always start with room temperature, well-mixed, clot free, EDTA anticoagulated blood (heparin is used for some exotic species). If the blood has been refrigerated, allow it to return to room temperature.
- The best slides to use for making blood smears are the premium, pre-cleaned kind; those with a frosted end also facilitate easy labeling. If you are not using premium pre-cleaned slides, you will need to wipe off each slide you plan to use for making the blood smear, including spreader slides, in order to eliminate glass grit and dust which can ruin your smear.
- Always mix the specimen immediately before making the smear by gently rolling the tube several times to ensure good cellular distribution, don't shake or invert.
- To transfer blood from the tube to the slide, fill a plain microhematocrit tube with blood by capillary action and place a finger over the end to prevent the blood from running out. Do NOT try to dab blood from the cap onto the slide or use a 1 ml plastic pipette; the microhematocrit tube will give you better control and, if you have a microhematocrit centrifuge, you can simply plug the clean end after making your slide and spin the tube for your hematocrit and total protein.
- Place an approximately 4mm diameter drop of blood on one end of the slide. If there isn't enough blood in the microhematocrit tube to produce the required droplet of blood, DO NOT repeatedly tap the tube onto the glass to try and get enough blood out; go back and get some more from the EDTA tube.
- Holding the spreader slide at an approximately 30-45° angle, back into the drop of blood and, as soon as you see the drop spreading along the edge of the spreader slide, push the spreader slide forward in a smooth, moderately fast motion; apply only enough

A well made blood smear. The classic blood smear has a thumb print appearance. A well developed feathered edge is apparent. The dense body of the smear takes up most of the slide and there is a thin counting area which blends with the feathered edge and the dense body.

pressure to keep the spreader slide on the glass, excessive pressure will push too much blood forward without allowing development of a good body and monolayer; you should be pulling the blood along the slide, not pushing it.

- If the blood is very thin (severe anemia) you may need to increase the angle of the spreader slide to avoid going off the end of the slide.

A good quality smear has the following features;

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

Poor quality slides are typically cause by excessive downward pressure, a slow spreading motion, or wobbling of the spreader slide on the surface of the smear. Slow spreading motion tends to cause long, thin smears that lack a dense body, thin monolayer and well-developed feathered edge. The leading edge may have a line of blood instead of a thin, feathered appearance from pushing the blood instead of pulling it. Often there is streaking in the smear. Linear lines arranged horizontally to the leading edge are called hesitation marks and they indicate hesitation in the forward motion. Excessive downward pressure will produce short slides with hesitation marks and a poorly developed feather edge and monolayer region. Wobbling is often due to inexperience or trying to exert pressure rather than letting the spreader slide rest on the surface of the smear. If you are having trouble making quality slides, it can be helpful to have an experienced individual spot your technique
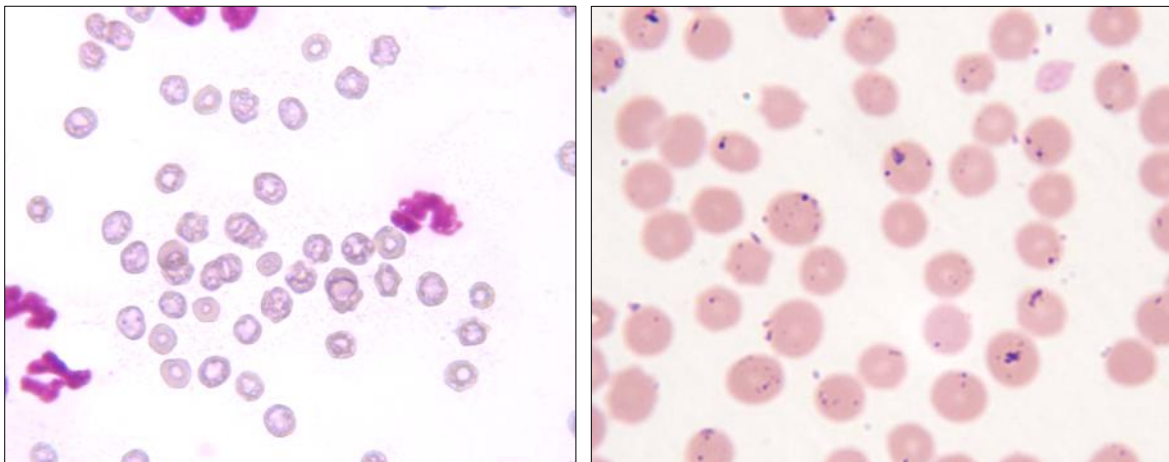
Slide staining
Romanowsky-type stains give good nuclear and cytoplasmic detail. Red blood cells stain red-orange, nuclei stain blue-purple and cytoplasm stains blue to pink. Most commercial laboratories use some form of Romanowsky-type stain (e.g. Wright - Geimsa) and these stains give excellent result but tend to be fussy. Quick Romanowsky type stains (such as Diff-Quik) are modified versions that are inexpensive, robust, fast and easy to use and generally give very good results. However, note that mast cell and basophil granules may not stain. I recommend the standard 6/6/6 dipping protocol for blood smears; dips should be slow (1-2 seconds each) and smooth. Some people prefer to add 2-3 extra dips when examining a smear for red cell parasites as this makes the organisms stand out more, but this shouldn't be done routinely because it imparts an excessive blueness to the slide making toxic change and polychromasia difficult to recognize.

Staining problems. The most common problems arising from use of rapid stain are 1) poor sample fixation 2) under-staining of the specimen 3) poor staining from use of weakened or exhausted stain or fixative and 4) stain contamination 5) excessive stain precipitation.

- Poor sample fixation. You can't over fix a slide, but you can definitely under fix one, and under-fixed cells will lyse and fail to stain adequately. Once you move from the fixative solution to the stain solution, an under-fixed slide can't be improved. For very thin cytology smears and for blood smears, 5 or 6 dips in the fixative solution should be adequate, but for thick cytology smears, up to 120 seconds may be required for adequate fixation. When in doubt, fix them a little longer.
- Under-staining of the specimen. This occurs when there is inadequate contact with the stain solutions. It can also occur if you attempt to stain several slides at once and the specimen

surface of one slide is covered by another slide. As long as a slide has been adequately fixed, a poorly *stained* slide can be put back into either stain solution for additional time, thus a poorly stained specimen can gradually be stained to the desired color intensity. An over-stained slide can be soaked in fixative solution until stain is removed and re-stained, although I have often found that with rapid stains, results are not as good as getting it right the first time.

- Poor staining due to weakened/exhausted stain or fixative. Stain solutions do weaken with repeated use, and prolonged exposure of the fixative to air at room temperature can result in impaired staining from formation of degradation products. How often you need to change the stain will depend on how many slides are put through it. The best advice is to keep an eye on the quality of the slides; when staining is not as good as typically seen, it's time to change the solutions. Signs that your stain is weakening include overall pale staining, especially pale blue nuclei that lack sharp chromatin detail. Never top off a weakened stain, it must be changed! Also remember that stain, even when kept in closed containers, will eventually go bad. If you don't use a lot of stain, purchase smaller containers to avoid losing money on stain that goes bad before you can even use it.

- Contamination. Stain contamination occurs when 'dirty' specimens such as fecal smears, ear scrapings, skin cytology or material from abscesses are stained in the same solution as 'clean' specimens (blood films, most cytology specimens). The easiest way to avoid this is to set up two staining stations, one for clean samples and one for dirty samples. Contamination of the fixative with water can produce refractile 'water artifact' on slides. Slides should be completely dry before staining (use of a gentle amount of warm air from a hair dryer can facilitate this), slide holders should be dried before slides are loaded, and stains should be tightly covered when not in use.

- Stain precipitation. This appears as fine, dark blue-purple stippled material on the background of the slide and on the cells. It makes identification of small hemoparasites a nightmare. Typically it's associated with the development of a green sheen on the surface of the dark blue stain solution; changing the solution for new typically eliminates the problem.

Staining artifacts. Left; refractile water artifact in red cells. Right; stain precipitate, image courtesy of Ms. Tillie Laws.

Microscopy

You will need the standard objectives for blood smear review; 10x, 40x and 100x oil immersion. Remember, if you are using a 40x objective, you will need to place a coverslip on top of the slide to get a sharp focus; this objective is designed to be used with a cover slip and without one, everything looks a little fuzzy. A 50x oil immersion objective is expensive ($800-1000), but is very useful for hematology. It negates needing to use a coverslip and allows you to move seamlessly from low power

to 100x without worrying about oiling the 40x. It also magnifies everything more than the 40x which is helpful for morphology and cell evaluation.

Always adjust the microscope for Köhler illumination when you get ready to look at blood smears or cytology slides. It is done to ensure optimal lighting of the slide. Examining a slide with a microscope adjusted for urine sediments or fecal wet-mounts is a waste of time. The procedure is as follows;
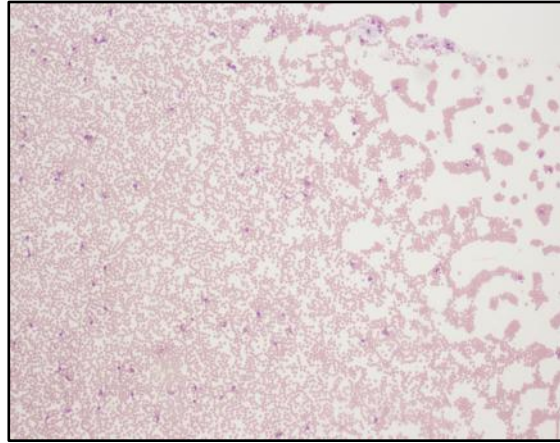
1. Focus on a slide at 10x
2. Close down the field diaphragm; this is located at the base of the microscope where light comes out. Look into the microscope while you close it down, you should see the light reduced to the center of the field as you close it. If your microscope is really off, instead of seeing a central circle of light, everything may just get really dim. Inexpensive microscopes may not have a field diaphragm. If you are trying to adjust a microscope that doesn't have one, skip the next step and simply raise the condenser *almost* all of the way up.
3. Adjust the condenser vertically via the condenser focus knob until the circle of light has a sharply defined edge. The condenser is portion of the microscope underneath the stage and there is a knob located on one side that moves the entire unit up and down. Usually the condenser must be moved up because someone has lowered it to look at urine sediments or fecal wet mount preps.
4. Center the ring of light using the silver centering screws on the condenser
5. Open the field diaphragm so that the entire field is flooded with light.
6. Open the condenser diaphragm about ¾ of the way. **NOTE:** This is actually a small fib, but it is easy to remember and works pretty well. If you want to learn more about microscopy including the technically correct last step in Köhler illumination, visit http://microscopy.fsu.edu/primer/index.html for more information.

Systematic smear evaluation
Be systematic and thorough when evaluating blood smears. It actually makes the procedure more efficient and you are less likely to overlook a major finding. You might find it's beneficial to start a small slide collection. Include a few examples of normal smears from the species you work with and examples of anemia, leukopenia and leukocytosis (especially useful if you have the actual counts to correlate with the appearance on the slide) and good examples of atypical/abnormal cells and morphology changes. These slides can be used as references or to train new individuals.

1. Examine the entire feathered edge. You are looking for large things because very large items often end up on the feathered edge. Common things include platelet clumps and microfilaria, less common things include blast cells, mast cells, and macrophages, rare things include megakaryocytes or schizont laden macrophages in Cytauxzoon infections. You also want to see if a disproportionate number of the WBCs have ended up on the feathered edge – this happens with poor smear technique and is important if you are estimating cellularity vs. using a machine count.
2. Estimate/confirm cell counts. If you don't have a hematology instrument, you will need to estimate WBC density and the platelet count, and even if you do have an analyzer, you should still correlate the machine count with the slide. Estimations are crude, but are useful for detecting significant changes. For WBCs, examine the slide on 10x in the monolayer (see #3 for how to find the monolayer) region. Normal animals will have anywhere from 18-50 cells per 10x field in health on a well made blood smear. Each WBC corresponds to about 330 cells/µl. While typically assessed via a PCV or with an analyzer, if needed, the RBC density can also be estimated. Using the 10x objective, start at the middle of the feathered edge and move back in a straight line through the monolayer counting area and into the body of the slide. In a normal animal, the RBCs should quickly become thickly piled up after leaving the monolayer region, but in anemic animals, the cells will never pile up as well, and the counting

area will be extended. Anemic animals will also have a paler blood smear grossly, so it's beneficial to look at the smear against a white background before putting it on the microscope.

3. Find the monolayer. Start at the very edge of the feathered edge on 10x. Move back towards the body of the slide. At the leading edge of the smear, you'll notice the blood cells break into little pools of cells with white spaces between them giving a reticulated pattern. As you move away from the feathered edge and into the monolayer, the white spaces disappear and the red cells form a smooth monolayer. Generally, it takes about 2-4 10x fields to get from the feathered edge into the monolayer. When you are doing your WBC and RBC morphology, you need to stay in the monolayer region. If you wander too close to the feathered edge, you'll see the reticulated pattern forming and if you wander too deep into the smear, you'll see the red cells start piling up on each other.


The transition zone between the feathered edge on the right and the monolayer on the left. Near the feathered edge, the red cells and white spaces for a reticulated pattern. As you move deeper into the smear, white spaces disappear as the monolayer forms

4. Examine the WBCs. Classify 100 cells for a differential count. It's often useful to just spend a few minutes looking around at what cells are present before you do a differential so you can decide who's who. Note any major morphology changes (toxic changes, reactive lymphocytes) and any abnormal cell populations (blast cells, immature neutrophils).

5. Examine the RBCs. Give an estimation (rare, mild, moderate, marked) of anisocytosis, poikilocytosis and polychromasia. Note that a well stained blood smear is needed to clearly see polychromasia. For poikilocytosis, look to see if there's anything specific such as acanthocytes or spherocytes.

6. Estimate or confirm the platelet count. **Remember to check the feathered edge for platelet clumps** as these will invalidate a low count. Using the 100x objective, average the number of platelet over several fields in the monolayer. Multiply this number by 20,000 to give a rough estimate of the platelet count.
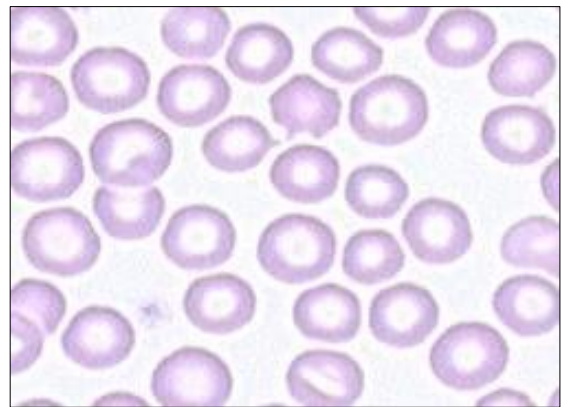
Red cells
Color changes.

- Polychromasia is recognized as larger, slightly bluer-staining red cells and is an excellent indicator of a regenerative response. Note: you will have a hard time identifying polychromasia if your smear is over-stained. Polychromasia is typically semi-quantified as rare, slight, mild, moderate or marked (can use similar criteria as with morphology quantification, see below).
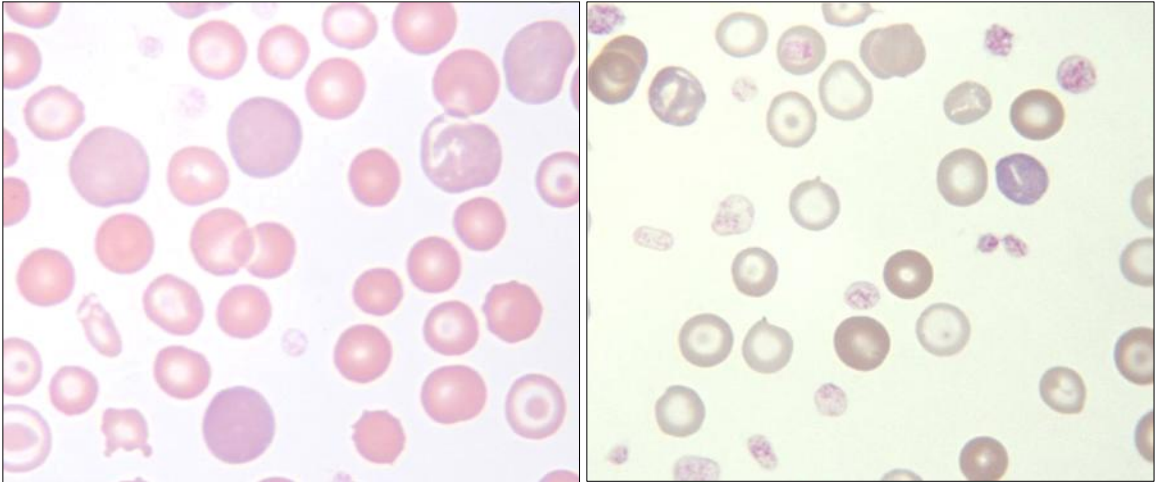
Red cell color changes. Right; polychromatophilic RBCs are present. These cells are larger and more basophilic than normal RBCs. This image show moderate polychromasia. Left; hypochromatic RBCs. The area of central pallor is increased and the cells, overall, are paler staining. Note a couple of polychromatophilic RBCs are also present. Images courtesy of Ms. Tillie Laws.

- Hypochromasia indicates iron deficiency and is recognized by increased central pallor AND pale color. This is recognized in dogs more often than cats. This is different from 'punched out' RBCs in which there is a distinct, round, pale central area that is sharply delineated from the rim of hemoglobin. Punched out cells have a wider rim of hemoglobin than do hypochromic cells, and the hemoglobin color is normal, not pale. Note that in iron deficiency, there often is a lot of poikilocytosis present as well.


Red cells with a 'punched out' appearance; the area of central pallor appears enlarged and is sharply delineated from the cytoplasm. This has no significance and should not be confused with hypochromasia; the rim of cytoplasm is still larger than is seen in hypochromic RBCs and the color of the cytoplasm is robust.

Morphology changes
- Changes can be quantified as rare (<1/100x field), occasional (1-3/ 100x field), few/mild (4-10/100x field), moderate (10-25/100x field) or marked/many (>25/100x field). In practice, these are estimated rather than actually counted.
- Acanthocytes. These are cells with unevenly distributed cytoplasmic projections, and are most commonly seen in feline hepatic lipidosis or in dogs with hemangiosarcoma, especially when it involves the liver.

- Schistocytes. These are small, irregular fragments of cells and are most commonly seen in hemangiosarcoma, microangiopathies, DIC and iron deficiency.
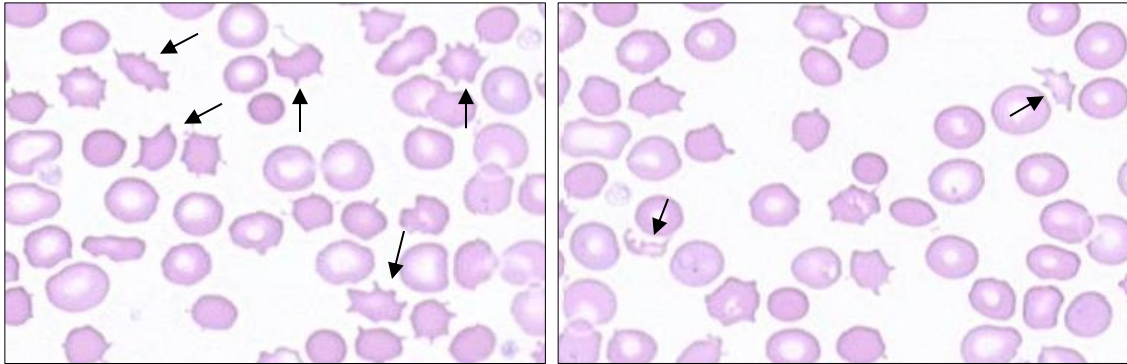

Many acanthocytes and schistocytes. On the left, several acanthocytes are indicated by the arrows. On the right , two schistocytes are indicated. Acanthocytes have irregularly spaced cytoplasmic projections that are typically blunted and may have a knob-like end. Schistocytes are irregular, torn pieces of RBC cytoplasm formed by sheering of cells.

- Spherocytes. These are red cells that are round instead of flat and most commonly indicate IMHA, but can also form after removal of Heinz bodies by the spleen and low numbers are noted in fragmentation anemia. Because they are rounded, they appear smaller and darker than normal red cells and there is a lack of central pallor. Spherocytes are best appreciated in dogs due to the prominent central pallor in the normal canine RBC, and are more difficult to detect in other species. Be careful not to interpret RBCs along the feathered edge as spherocytes; cells in these areas (where there are large open spaces) tend to round up and lose their central pallor normally.
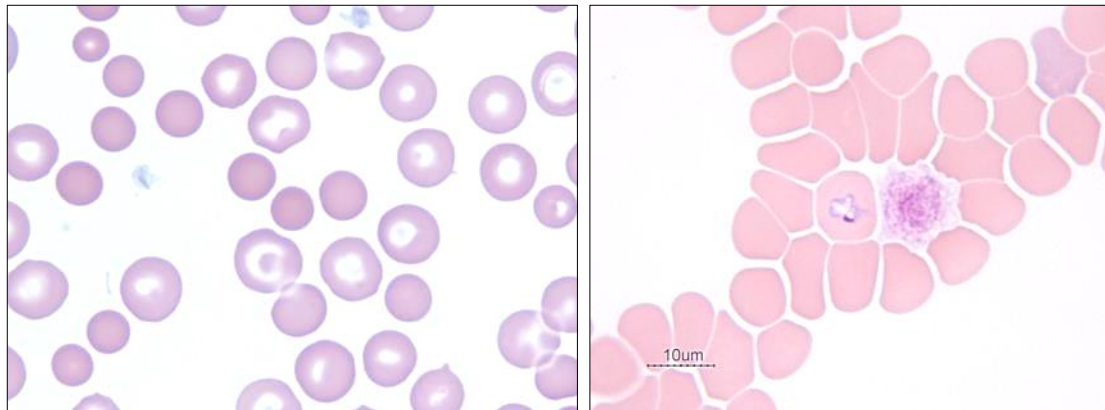

Right; an example of spherocytes in a dog. Spherocytes classically are small, round cells that lack central pallor and appear darker red than surrounding blood cells. Sometimes, when they have all the features of spherocytes but do not appear darker than surrounding cells, they are referred to as 'pre-spherocytes' or 'imperfect' spherocytes and may also indicate immune mediated targeting of RBCs. Left; RBCs from the feathered edge of a canine blood smear. Note that the cells appear to have lost their central pallor – don't mistake RBCs without central pallor on the feathered edge for spherocytes, this is normal. This dog also has a large platelet and, in the RBC in the center, a *Babesia canis* piroplasm.

- Heinz bodies. These indicate oxidative damage to hemoglobin, and large numbers are seen with oxidative hemolytic anemia. They appear as small, roundish structures can protrude from the margin of the cell as a pale structure, or appear as a small pale dot near the edge of the cell. Cats often develop large Heinz bodies when it's the result of an oxidative drug or plant; Heinz bodies seen in sick cats (renal disease, lymphoma, hyperthyroidism) tend to be smaller and present in lesser numbers.
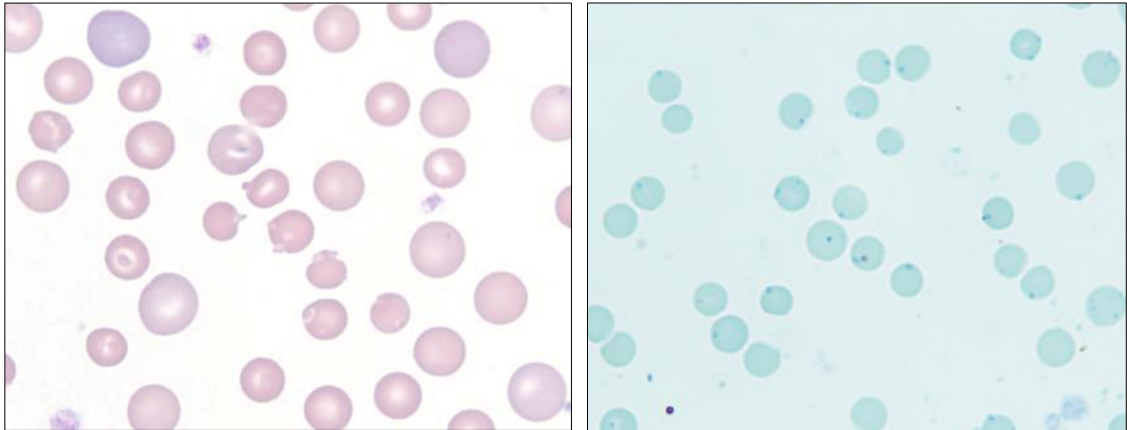

Heinz bodies. Left; on Romanowsky stained slides, Heinz bodies appear either as small projections from the margin of the cell, or pale stained regions on the interior of the cell. Right; with new methylene blue staining, Heinz bodies are basophilic blue dots.

- Blister cells and keratocytes. Blister cells are red cells with a round, clear blister at the edge of the cell. When the blister ruptures, one or two thin remnants of cytoplasm are seen and it's then called a keratocyte. These shape changes indicate oxidative damage and are most common in iron deficiency, but can also be seen in any cause of oxidative damage including toxicities (e.g. onions) or drugs. Eccentrocytes are a related shape change also indicating oxidative damage. They form the RBC cell membranes stick together and are identified by seeing cells with a pale region where membranes are fused and a darker region where all the cytoplasm has been pushed to one side. These can look like spherocytes, but careful examination will ensure you see the pale area of fused membranes.
- Echinocytes. These are cells that are covered with small, blunted to sharp, evenly distributed spicules; they look sort of like a sea urchin. This can be a drying artifact, in which case they will be unevenly distributed on the slide and are called crenated cells. When real, they are associated with renal disease, severe electrolyte abnormalities and snake-bite envenomation. With snake bites, virtually all cells are affected and the spicules are small and very sharp.
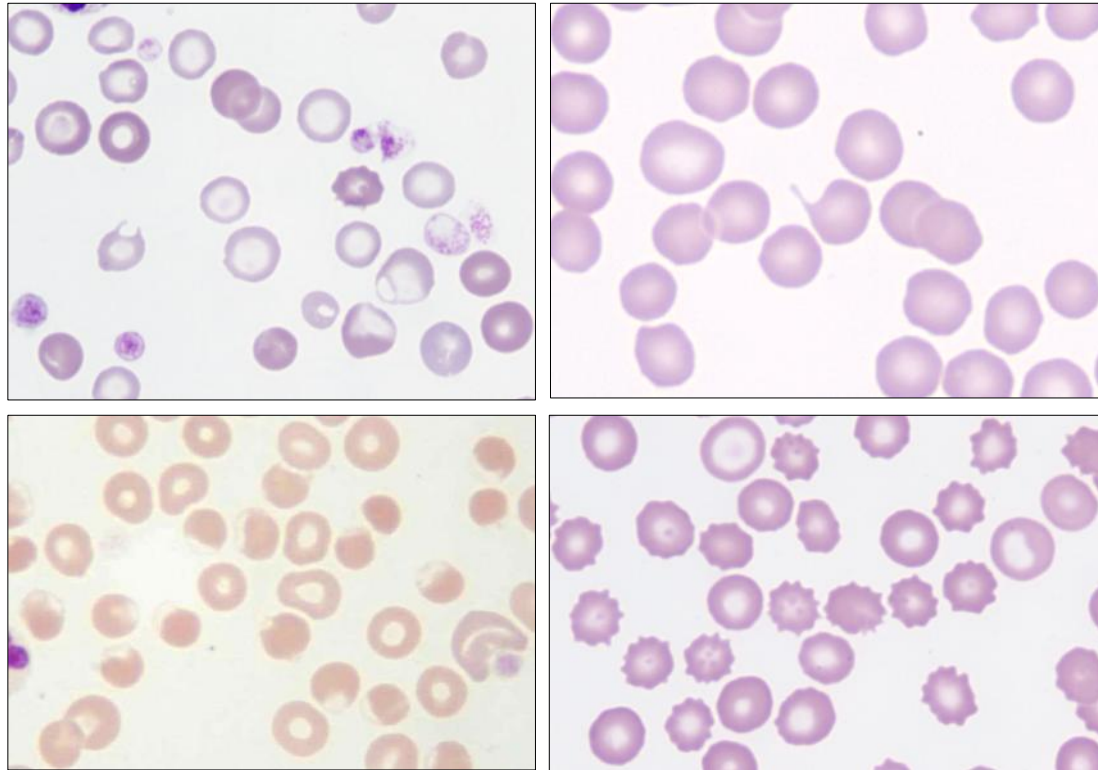
Top left; a keratocyte (left arrow) and a blister cell (right arrow). This dog is also hypochromic. Top right; a keratocyte with only one projection – some people refer to these as 'apple-stem' cells. Bottom left; eccentrocytes characterize by fusion of the RBC membranes creating a pale region (image courtesy of Ms. Tillie Laws). Bottom right; echinocytes characterized by small, evenly spaced spicules.

Agglutination and rouleaux. Agglutination indicates IMHA and appears as grape-like clusters of RBCs. Often the best places to look for agglutination are just behind the feathered edge or between the monolayer and the body of the smear. It's also easier to spot on 10x vs. 40x or higher. High protein levels can also cause red cells to clump and appear agglutinated, so if you think you see it, you need to do a saline dispersion test to confirm. Take 1 drop of patient blood and mix with 2-3 drops of normal saline. Tilt the slide back and forth to get the fluids mixing and moving, and then look at it under the microscope right away on 10x, while the cells are still in motion. You'll need to drop the condenser down or stop down the condenser like you would for examining fecals or urine sediments since the blood is unstained. Look at the cells as they fly by. If the cells are generally individualized and only rarely in twos or threes, then it's negative, but if you see whole clumps going by, it's positive. Rouleaux occurs when RBCs are stacked like coins and is best appreciated on 10x as well and is most prominent where the monolayer starts blending into the body. Some rouleaux is OK in cats, horses always have it. Increased rouleaux typically occurs in inflammatory conditions or with hyperproteinemia, including animals with gammaglobulinemias (multiple myeloma, some lymphomas). It will disperse with saline.
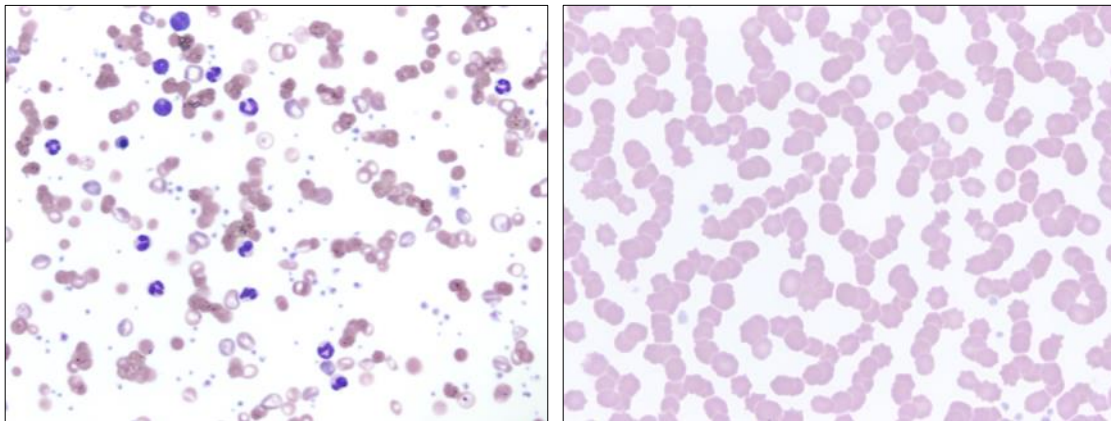
Left; canine blood smear showing agglutination. Cells are present in small clusters that are easiest to appreciate on low power. Right; normal equine blood smear showing the 'stacked coin' appearance of rouleaux.

Red cell parasites

- *Mycoplasma. M. haemocanis and M. haemofelis* appear as small, epicellular bacterial organisms on RBCs. They can form small rings, dots, and linear chains. Cats can also be infected with *M. haemominutim* which is less pathogenic and also smaller and more difficult to identify. When *Mycoplsma* is seen on the very periphery of the cells, it's possible to see a slight indentation of the cell membrane. The organism can be associated with agglutination and, in cats with *M. haemofelis,* there is typically a strongly regenerative hemolytic anemia present unless there is a concurrent immunosuppressive disease or condition (FeLV for example). There are a few things to keep in mind with this parasite. The first is that cytologic detection has low sensitivity compared with PCR, so not seeing the organism is no reason to exclude it. Second, appearance of the organism in peripheral blood, especially in cats, can be
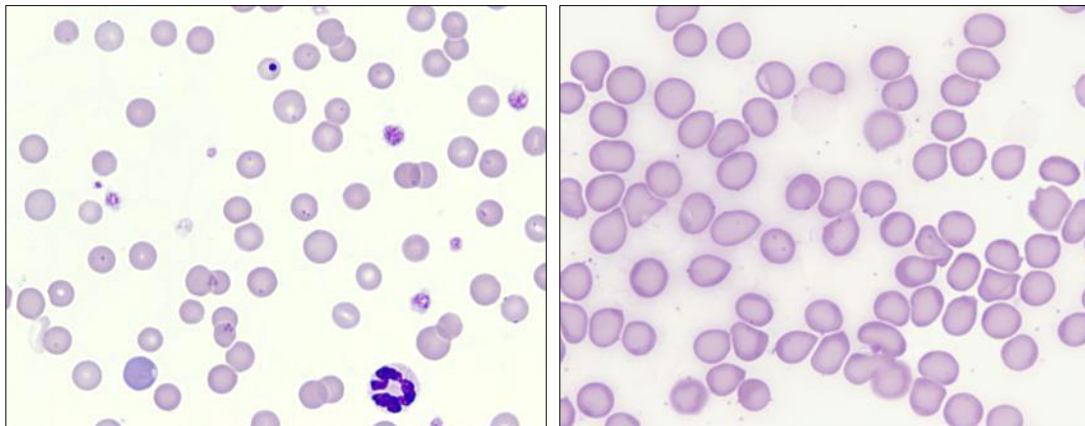
Mycoplasma. Left; *M. haemofelis*; note the extremely small size of the organisms. Left; *M. haemocanis*. Note the organisms in the background that have fallen off the RBCs. Clean stain is essential when looking for this organism

episodic, so it can be missed. Third; once the infected RBCs sit in EDTA for awhile, the organisms start to fall off the cells, so a freshly made smear soon after collection is best. Fourth; if your stain has a lot of precipitate or your fixative is contaminated with water causing refractile water artifact, this organism can be very difficult to distinguish from debris, so use clean stain.

- *Babesia canis. B. canis* is a protozoal organism that forms large, piriform (tear drop) shaped merozoites in RBCs and these may be paired. They generally are about 3-5 µm in size with a dot-like nucleus and pale blue cytoplasm with a distinct cell wall. There are subspecies of *B. canis* and, in the US, the most common is *vogeli* which also happens to be the least pathogenic. The same is not true of the other subspecies which can cause more severe disease. Most cases in the US represent subclinical carrier states, although this organism can cause more severe disease in young animals, animals that are heavily infected and in those with underlying conditions. *Babesia* can be detected by PCR. Examination of peripheral capillary blood from the ear or nail bed may concentrate the organisms making it easier to identify them. Although *B. canis* is the most common large form *Babesia* seen in dogs, it's not the only one; other species have been detected.
- *Babesia gibsoni. B gibsoni* is a small form of *Babesia* and is much more pathogenic; acute infection can cause severe hemolytic anemia, thrombocytopenia and a shock-like syndrome. It's also been linked to dog fighting or bites from dogs involved in fighting. Dogs surviving the acute phase can show non-specific hematologic findings but often have mild anemia and thrombocytopenia. Although this organism is also detectable by PCR, making a timely and accurate diagnosis can be more urgent, thus identification of infection on a blood smear is of significant value. These organisms are much smaller than *B. canis*; they are typically 1-2.5 µm in size with a very small dot like nucleus and a ring shaped or safety pin shaped membrane. The membrane is thinner and more delicate and the nucleus smaller compared with *B. canis* making them more difficult to see. As with *B. canis*, capillary blood from the ear or nail may concentrate infected RBCs. There does appear to be different isolates of this organism, and there may be different organisms with similar cytologic appearance, but all are associated with more severe disease. Staining slides with quick stains such as Diff Quik and staining them more intensely with the second stain solution (6-6-9 or 6-6-12) can facilitate identification. Clean stain is essential.
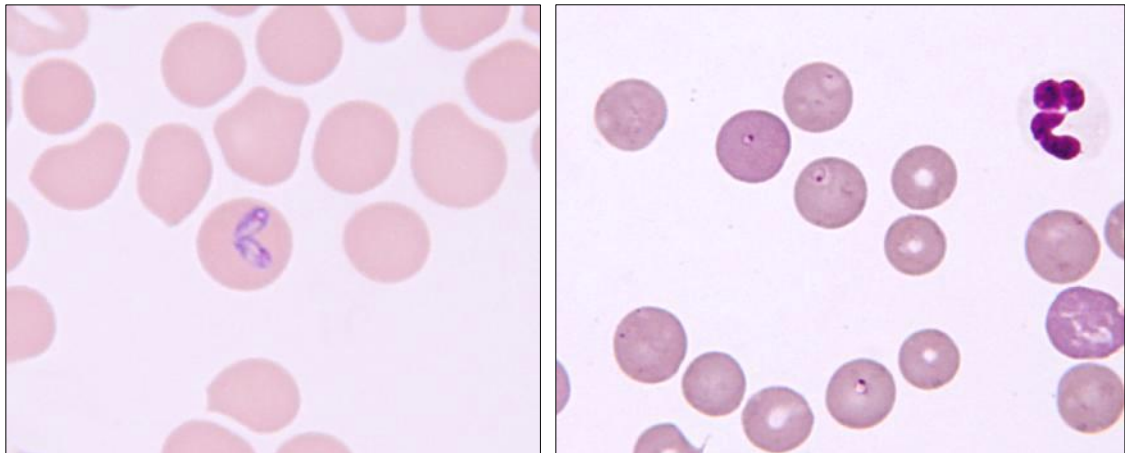
Babesia. Left; *B. canis* showing the classic piriform (tear) shaped organism. Here they are paired and you can clearly see the nucleus, cell margin and the pale cytoplasm. Note that they are relatively large and easy to see. Right; *B. gibsoni*. Notice these organisms are very small and difficult to see. They are ring shaped with a dot like nucleus. This slide was stained with a quick stain (Diff Quik) with extra dips in solution II (dark blue) in order to enhance detection of the parasites.

- *Cytauxzoon felis. C. felis* is a devastating protozoal disease of cats that is often, but not always, fatal; timely identification with aggressive treatment is successful in a significant number of cats. Although PCR is available, it is not really of much diagnostic utility due to the rapid progression of the disease. Identification of organisms by light microscopy remains the best means of diagnosis in the clinical setting. Parasitemia is variable necessitating a thorough

search for organisms. The organisms are very small (about 1-1.5 µm), ring or safety pin shaped structures with a very small dot-like nucleus. Similar to *B. gibsoni*, over-staining with solution II of the quick stains can facilitate identification and clean stain is essential. Unlike *Babesia*, *C. felis* has a tissue stage in addition to a red cell stage, and it is believed that proliferation of the tissue stage is responsible for the shock-like syndrome that cats develop. The tissue stage develops before the red cell stage, so suspect cats that are negative for the organism on peripheral blood should have tissue aspirates performed to look for the tissue stage. The tissue stage consists of hypertrophied macrophages with developing schizonts within their cytoplasm. The cells are large with very large eccentric nuclei that contain prominent nucleoli. The cytoplasm is distended with developing schizonts. Early in development, the schizonts appear as pale blue lobulated clouds of material. As the schizonts develop, the material becomes mottled with magenta nuclear material. The latest stages can be identified as stippled material representing individual organisms within the macrophage cytoplasm. Although the organism can be found in almost every tissue, lymph node, spleen and liver aspirates have been the best tissues for anti-mortem identification due to their tendency to have high numbers of schizont stages and ease of aspiration. Schizonts are occasionally seen on the feathered edge of peripheral blood smears.
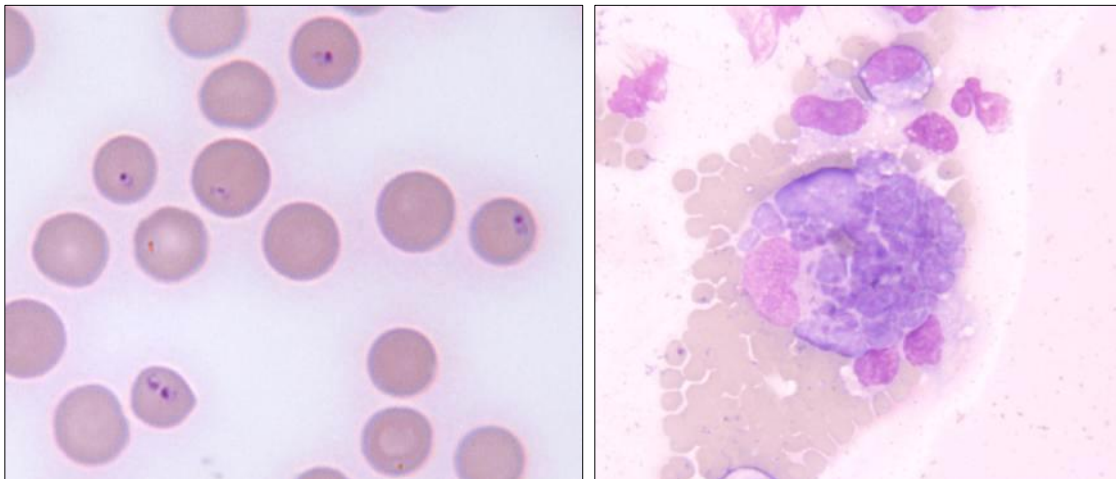

Cytauxzoonosis. Left; the typical appearance of the red cell merozoite stage. The organisms are very small with a ring or safety pin shape and a dot like nucleus. Like *B. gibsoni*, over-staining with solution II of the quick stains will make them stand out more. Right; a schizont laden macrophage on the feathered edge of a smear. The cells are very large with large, hypertrophied nuclei and cytoplasm that is distended with developing organisms. In this case, the schizonts appear as a basophilic, lobulated mass.

Nucleated red cells. Nucleated RBCs are important to identify for a couple of reasons; 1) they can be present for pathologic reasons and 2) they can falsely elevate total WBC counts if present in high numbers. Some hematology analyzers attempt to identify nRBCs with variable success. None consistently identify them and most will include them in the lymphocyte and / or monocyte counts. In the NCSU Clinical Pathology Laboratory, we correct the total WBC count whenever 4 or more nRBCs are identified during the manual differential cell count. By convention, most people keep track of nRBCs seen per 100 WBCs counted, but some laboratories will report them out as total counts per µl of blood, similar to WBCs. The formula to correct total WBC counts for nRBCs is:

Corrected WBC count = [(Uncorrected WBC count) x 100] / [(nRBCs per 100 WBC) + 100].

Nucleated RBCs can be seen during intense regenerative responses but should be accompanied by marked polychromasia and should be in relatively small numbers. When present in high numbers or

when present without intense regeneration, underlying diseases or conditions affecting the bone marrow or spleen should be considered. Nucleated RBCs have grayish blue cytoplasm and a nucleus that varies from small, eccentric, deep blue and condensed (metarubricytes) to large with coarse chromatin and a thinner rim of more basophilic cytoplasm (rubricytes). Cats with myleoproliferative disease can have less mature stages; this is rare in dogs. Less mature nRBCs can be mistaken for lymphocytes, but compared with a lymphocyte, they have coarser chromatin, more cytoplasm and the cytoplasm is more grayish blue; lymphocytes typically have only a small amount of cytoplasm visible on one side of the nucleus.
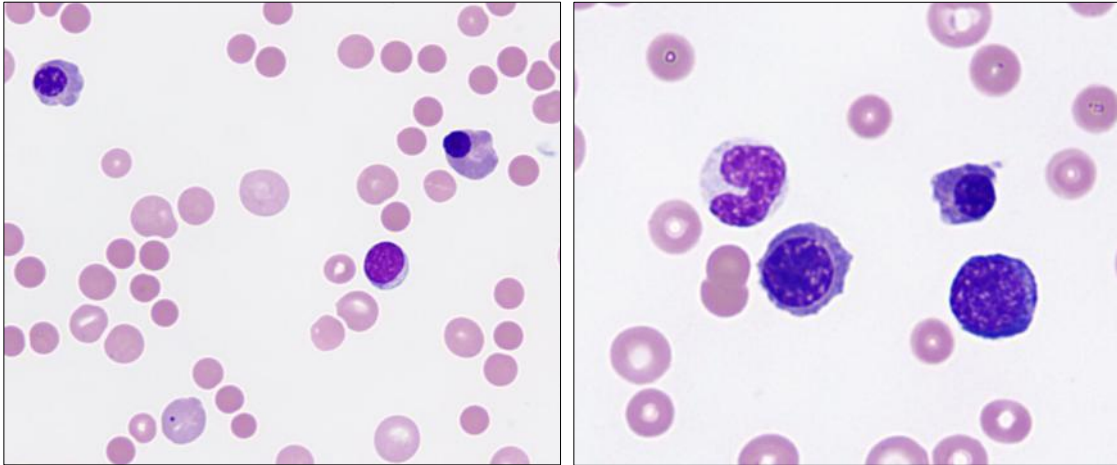
Nucleated RBCs from a canine blood smear. Left; lower power view of one metarubricyte (right), one late stage rubricyte (left) and a lymphocyte (bottom right). Metarubricytes have smooth, condensed nuclei that may be partially extruded from the cell. Late stage rubricytes have slightly larger nuclei that have not undergone condensation yet. Nucleated RBCs have a darker nucleus and more abundant gray-blue cytoplasm compared with lymphocytes which have lighter nuclei and a scant amount of paler cytoplasm. Note there are also many spherocytes present, a few polychromatophilic cells and one HJ body. Right; same dog showing one late stage rubricyte and two earier stages of rubricytes; earlier stages are larger with larger nuclei and less cytoplasm, but the chromatin pattern and cytoplasm color are similar. There is also a band neutrophil present.

White cell changes

Toxic change
Toxic change indicates acute, systemic inflammation and, when moderate or marked, is most closely associated with toxemia from bacterial infections. Slight toxic change is common in cats and slight to mild toxic change can be seen in a variety of disease processes that are not necessarily infectious (immune mediated disease, neoplasia, drugs) or bacterial (fungal disease, protozoal infection, viruses). Toxic change consists of 4 potential components;

- Cytoplasmic basophilia due to retained ribosomes. This is often the last change to resolve.
- Döhle bodies. These are small, cytoplasmic, angular, grey-blue aggregates of retained rough ER. Cats develop these commonly and a rare Döhle body can be seen in healthy animals.
- Cytoplasmic vacuolation due to granule dissolution, it's identified as cytoplasm with a frothy appearance. This is often seen in conjunction with increased basophilia but is a more serious manifestation of toxic change.
- Toxic granulation from retained primary granules. This is a severe change and is rarely seen in small animals (I've yet to see a convincing case in a dog or cat), is seen occasionally in large animals, and is most commonly seen in avian and reptile species. Toxic granules are pink-purple in color.

Grading of toxic change is subjective and depends on the features seen, the strength of the feature and the number of cells affected. In general, the level of importance is Döhle bodies < basophilia < vacuolization < toxic granulation, but, remember, toxic granulation is almost never seen in small animals. It's also important to remember when judging basophilia that 1) immature neutrophils (bands or earlier) are more basophilic normally so they are not the best cells to judge by and 2) over-stained slides make basophilia difficult to assess.
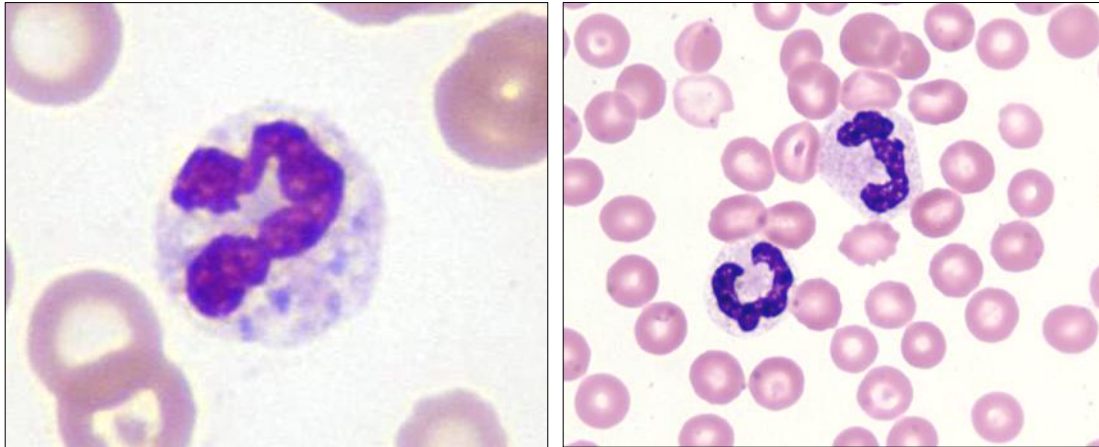
Canine toxic change. Left; a neutrophil shows increased basophilia, vacuolization and many small blue gray Döhle bodies (image courtesy of Ms. Tillie Laws). Right; similar changes but fewer, smaller Döhle bodies.

Left shifting
Left shifting is an important indicator of acute inflammation. Some hematology analyzers attempt to detect immature neutrophils, but none do it reliably. Band neutrophils are the most common stage identified and they are characterized by a horse-show shaped nucleus lacking the normal lobulation seen in mature cells. Note that there are differences between laboratories regarding the distinction between mature neutrophils and bands. Some labs are conservative and will only call a cell a band if it lacks any indentation at all (must be smooth horse-shoe shape with parallel cell walls), while others are more liberal and will call bands even if there is some indentation as long as it is mostly horseshoe shaped as lon. Less mature forms than bands (metamyelocyte stages and earlier) are characterized by a more bean-shaped nucleus that, in very immature stages, may totally lack any indentation.
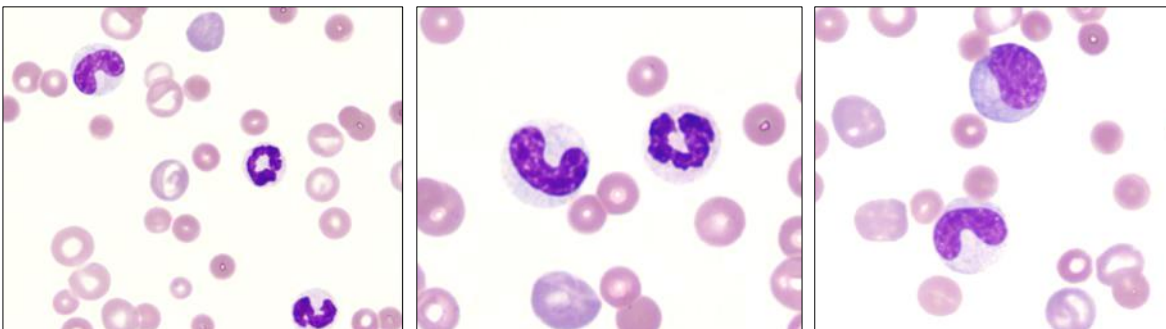
Left shifting. Right; a band is present at the top, in the middle is a mature segmented cell and at the bottom there is a non-filamentous segmented cell meaning a cell that is midway between being a mature segmented cell and a band. Middle; a band cell characterized by a smooth, evenly sized horse-shoe shaped nucleus compared with a mature cell. Left; on the bottom is a band cell while on top there is an early metamyelocyte, the stage before bands.
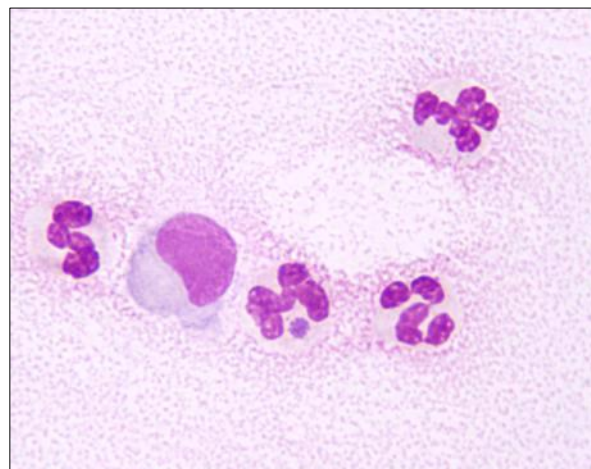
Sometimes people have trouble distinguishing bands or earlier stages from monocytes. In general, monocytes have deeper blue cytoplasm, often have a few medium sized vacuoles (larger and more distinct than vacuoles seen in toxic change), and even when the nucleus is 'band-shaped', they are more likely to have bulbous, flared ends whereas the ends of the band nucleus is consistently similar in size to the rest. It's also helpful to compare the chromatin of the suspect cell to that of the neutrophils you are sure of – a band will have a similar but lighter chromatin pattern.
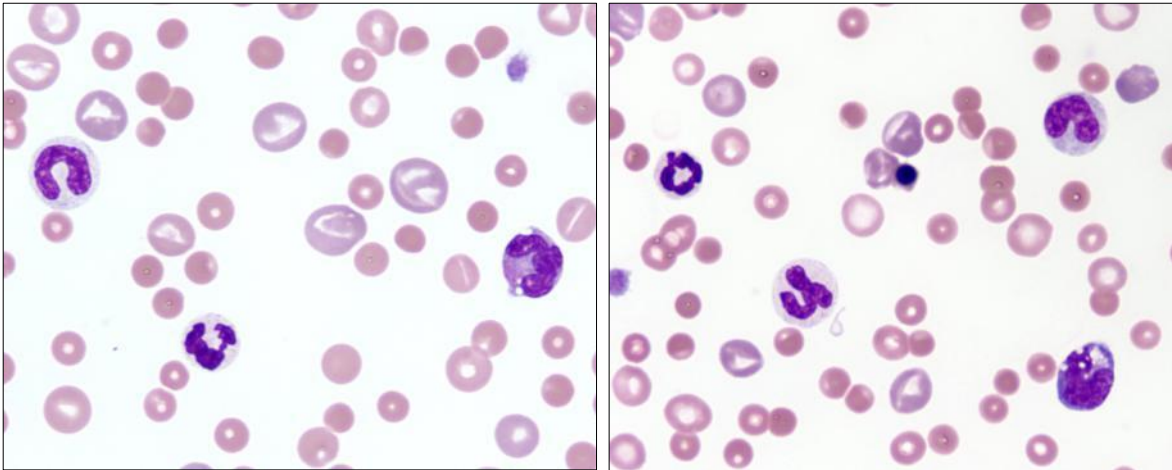
Monocytes vs. bands. Monocytes have darker cytoplasm, often have medium sized discrete vacuoles and a more variably shaped nucleus; even when 'band' shaped, the end of the nucleus are often expanded or flared. In addition, comparison of the chromatin of a monocyte to that of neutrophils and bands will reveal differences in color and texture. Left; from left to right are a band, a mature segmented cell and a monocyte. Right; from left to right are a mature segmented cell, a band and an early band / late metamyelocyte (top) and a monocyte (bottom).

Ehrlichiosis/anaplasmosis

Canine granulocytic ehrlichiosis/anaplasmosis is caused by infection with *Ehrlichia ewingii* or *Anaplasma phagocytophila* (formerly *E. equi, E. phagocytophila* and Human Granulocytic Ehrlichial Agent). Although diagnosis is often made via serum titers or PCR, it's a shame to miss an acute infection detectable on a blood smear. Also note that *E. ewingii* has a propensity for causing suppurative polyarthritis and the organism may be detected in joint fluid smears from acutely infected patients. The morulae appear as pale blue to lavender, round, intracytoplasmic structures with a coarsely clumped or stippled

E. ewingii in a canine joint fluid.

appearance. The color of the organism is different from the color of the cell's chromatin and this can help you distinguish an organism from a small nuclear lobe. People sometimes describe the organisms as looking like raspberries or asterisks. *Ehrlichia canis* is the cause of canine monocytic ehrlichiosis. Organisms reside in monocytes rather than granulocytes and while it's possible to detect them in peripheral blood, it's much less common. Concentrating cells via a buffy coat preparation or performing splenic aspirates may increase the chances of detecting this organism.

Blast cells

Blast cells in circulation can be an important indicator of underlying neoplasia, recurring neoplasia or stage of lymphoma. Note that with a severe inflammatory leukocytosis with a significant left shift, a few myeloblasts may be seen as part of the bone marrow response, but in the absence of these findings, or when blast cells are present in disproportionate numbers compared with other neutrophil stages, underlying neoplasia is a concern. Blasts are characterized by cells that are medium to large in size with pale chromatin, a visible nucleolus or nucleolar ring and, typically, a scant rim of cytoplasm. Other features may be present depending on the origin of the cell. Often small cytoplasmic fragments are also found in circulation. They are small platelet sized fragments of cytoplasm – they can be distinguished from platelets because they lack normal platelet granulation.
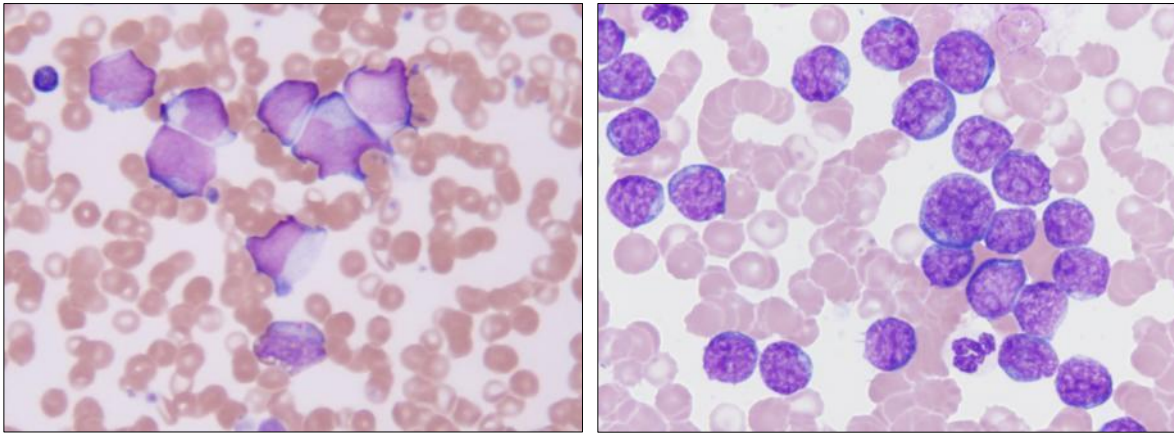
Blast cells. Left; the blast cells are large and variably shaped; they are seen molding to each other and to adjacent RBCs causing distortion of the cell shape. The chromatin is very pale and delicate. Nucleoli were faintly visible on higher power. Right; these blast cells are also large but exhibit less molding, have darker, coarser chromatin and more robustly visible nucleoli and nucleolar rings.

In small animals, neoplastic blast cells in peripheral blood are mostly lymphoid in origin, but acute myeloid leukemias do occur. Acute lymphocytic leukemia arises in the bone marrow and is typically associated with very high blast cell counts, but aleukemic and subleukemic leukemias with few or no blast cells in circulation can happen. Lymphomas arise in peripheral tissues and are associated with variable, but typically much lower, blast cell counts. Chronic lymphocytic leukemia is characterized by small to medium sized lymphocytes that lack overt blastic features. Sometimes ALL and end-stage lymphoma are difficult to distinguish.

Sometimes finding blast cells can be tricky. They are often very fragile cells so it's not uncommon for them to all rupture in the monolayer. Sometimes your first clue that they may be present is seeing very large ruptured cells in the monolayer and on the feathered edge of the smear. If you see this and can't find intact cells in the monolayer, try moving deeper into the slide along the lateral edges of the smear; often these areas are thin enough to see the cells but thick enough to protect them from getting broken. Another good place to look is the spot where you first put a drop of blood onto the slide to make a smear; often this area is also thinner but protected.

Mast cells
Mast cells can be found in circulation secondary to mast cell neoplasia or severe inflammation, especially of the GI tract. Often they are found on the feathered edge of the smear, but may be present in the monolayer when in high numbers. These are large cells with round nuclei that are filled with purple granules; in dogs, there is often a smudged purple 'halo' around the cells from partial degranulation, cats don't tend to do this. Don't mistake these cells for basophils. Basophils have band-

like nuclei and few dark blue granules (dogs) or many pale gray-blue granules (cats). Remember mast cell granules may not stain with quick stains.
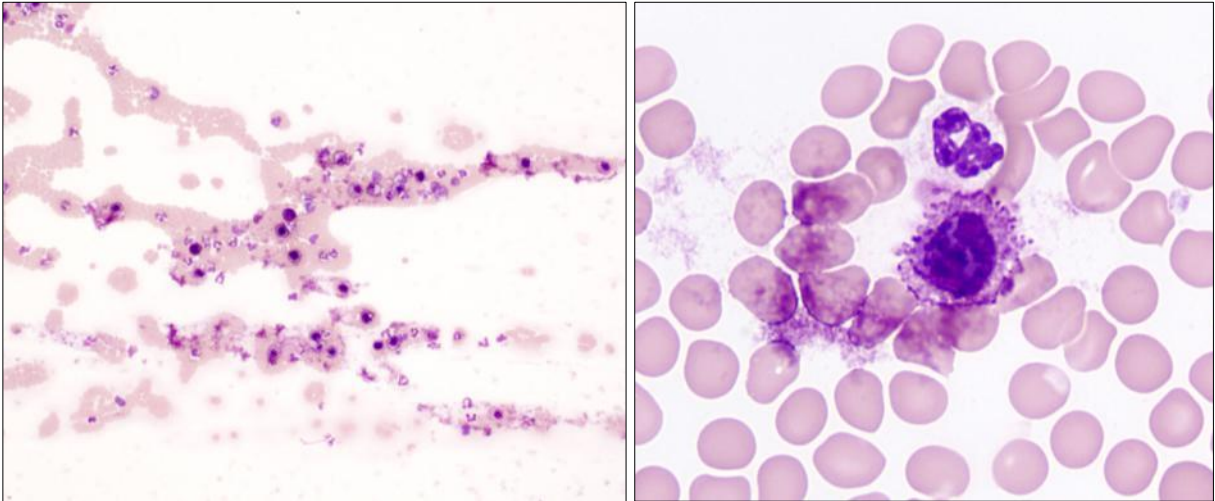

Mast cells. Left; low power appearance of mast cells on the feathered edge of a blood smear; cells appear as dark purple spots. Right; a high power image of a mast cell. These cells are characterized by a round nucleus and purple granules. Note the smudged purple 'halo' streaming away from the cell to the left; this is typical of canine mast cells seen on blood smears; cats do not tend to have a hale.

Platelets and miscellaneous

Confirm/estimate count

This is an important step, especially in animals with low platelet counts from a hematology analyzer. Most analyzers can't reliably detect platelet clumps and will give a falsely low number when clumps are present. Although this is more common in cats, it also happens in dogs. Clumps are easiest to identify on the feathered edge of the smear. They have a pale pink/blue stippled appearance. Platelet clumps always invalidate hematology analyzer or hemocytometer counts. If clumps are not present, you should perform an estimate in the monolayer region by averaging the number of platelets present in several 100x fields and multiplying that by 20,000. In general, if there is a large discrepancy between an analyzer count and your estimate (as long as there are no clumps present!), most people trust
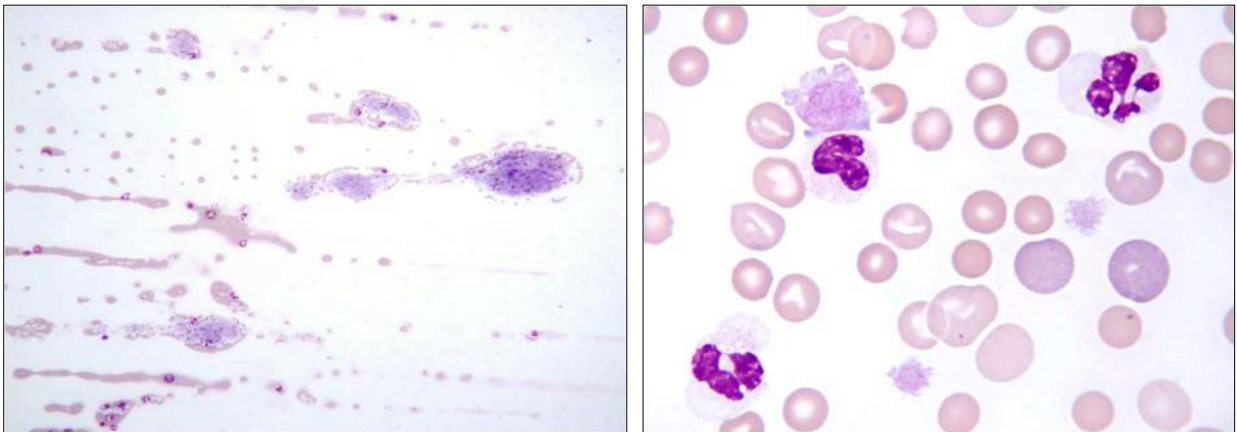
Left; low power view of platelet clumps which appear as pale blue material stippled with pink granules. Platelet clumps invalidate platelet counts and estimates. Right; a giant platelet is present above the band neutrophil. Giant platelets may not be counted correctly; they suggest a regenerative response to thrombocytopenia.

estimates more. Remember that impedance analyzers use size to distinguish platelets from RBCs so if there is overlap between the two; you can end up with an inaccurate platelet count. This is most common in cats.

Identify large platelets

Large platelets in thrombocytopenic animals suggest platelet regenerations. Some analyzers will provide you with a mean platelet volume which is interpreted similar to an MCV value, but you should still check a smear for evidence of large platelet in thrombocytopenic animals since an MCV may not detect low numbers of large platelets and because many analyzers have difficulty counting and sizing platelets when they near RBC size.

Microfilaria

Blood smear evaluation is not how heartworm disease is tested for, but in positive animals, it can give information about the degree of microfilaremia. Occasionally we detect microfilaria in dogs not suspected of having HW disease. Microfilaria are often found at the feathered edge of the smear, but could be anywhere on the slide. When present in low numbers, they are surprisingly easy to miss unless you do a low power (10x or 4x) evaluation of the smear.

High globulin levels

When globulin levels are significantly increased, the protein will be present as a pale pink background. In thicker areas of the slides, the protein background will fold over on itself as it dries creating protein crescents or 'finger-nail clippings'.
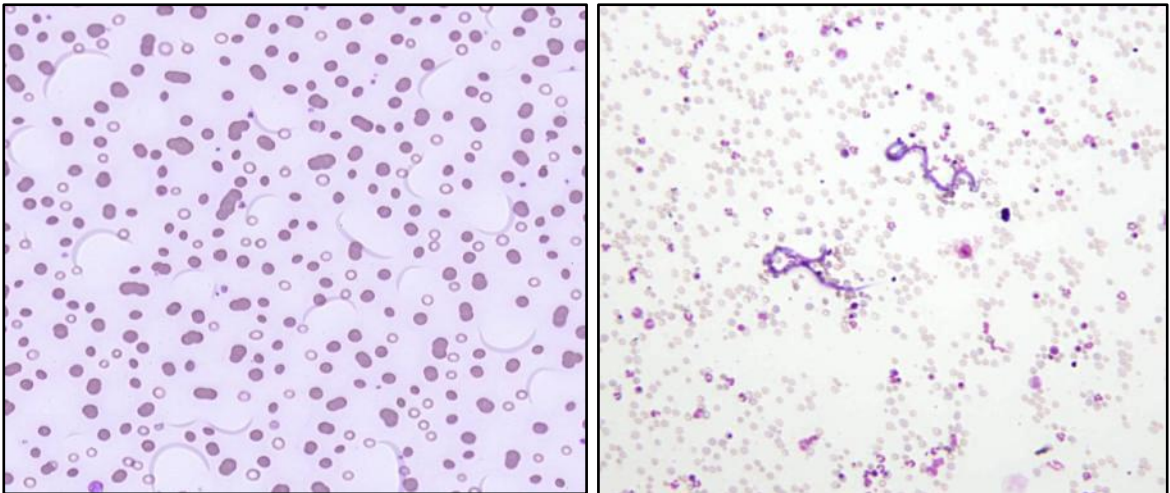
Left; blood smear from a cat with multiple myeloma and very high globulins. Note the many protein crescents or 'fingernail clippings' in the background. Don't mistake these for a parasite! Right; blood from a dog with heartworm disease and secondary IMHA. When present in low numbers, microfilaria may be easier to find on the feathered edge or deep in the smear.