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Haematology: The Avian Blood Film

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Evaluation of cell morphology in the stained blood film is an important part of haematology and the evaluation of the avian patient. Often, the stained blood film is the only component of hematology available to the avian veterinarian because of a small sample size. A single drop of blood can provide valuable information in the assessment of the patient.

A properly prepared blood film should not extend to the edges of the slide and will have a thick body that tapers into a feathered edge. The best cell morphology lies just behind the feathered edge in the monolayer area. It is difficult to examine cells in the thick part of the blood film because they superimpose over each other and the leukocytes appear rounded and are not able to expand and flatten out (making them all resemble lymphocytes). Examination of cells in the feathered edge area will reveal artifacts such as ruptured cells.

A blood film made from a drop of non-anticoagulated blood placed on a slide immediately after collection is preferred over a blood sample exposed to an anticoagulant. Anticoagulants, such as heparin and citrate, often affect the staining quality of the blood film. The anticoagulant EDTA (ethylenediaminetetraacetic acid) may cause hemolysis or the blood to clot in some species, such as birds in the crow family (corvids) rendering the sample useless. Once the film has been prepared, it should be dried immediately. In most settings, this is accomplished by waving the slide in the air; however, in high-humidity use of a commercially available slide warmer or a hair dryer set on a low (warm) setting held in front of the slide may be needed to properly dry the slide to prevent drying artifacts, such as excessive crenation of the red blood cells. Blood slides should be labeled with the animal identification information and the date. Romanowsky stains, such as Wright or Wright-Giemsa are commonly used stains for the evaluation of haemic cytology.

At low magnification (using a 10x or 20x objective), an experienced cytologist can subjectively estimate the leukocyte concentration on a blood film as being low (leukopaenia), normal, or high (leukocytosis) before examination of the cells using the 100x (oil-immersion) objective. A number of formulas for estimating the total leukocyte and thrombocyte concentrations from a blood film have been proposed; however, none are accurate or precise and should not be used in reporting leukocyte and thrombocyte numbers. The semidirect manual method that uses a Phloxine B solution for obtaining an absolute (total) leukocyte count is generally the preferred method in avian haematology. The morphology of the three major cell types (erythrocytes, leukocytes, and platelets or thrombocytes) is best evaluated at higher magnifications.

A differential leukocyte count is obtained by counting a minimum of 100 consecutively encountered white blood cells in the monolayer area of the blood film. For birds, the cells are classified as heterophils, eosinophils, basophils, lymphocytes, and monocytes to obtain a relative percentage for each leukocyte type. Cells not readily identified can be placed into a sixth category of "other." Abnormalities in leukocyte morphologies are noted.

Evaluation of erythrocyte morphology is also made using high magnification. Important erythrocyte abnormalities include changes in cell shape, color changes, presence of inclusions, and changes in the position of the cell nucleus.

Thrombocytes are evaluated under high magnification. Their numbers can be estimated as being adequate, low (thrombocytopenia), or high (thrombocytosis). In birds an average of 1-2 thrombocytes per oil immersion monolayer field is considered to be adequate. Lower numbers may indicate a true thrombocytopenia or excessive platelet clumping; the latter of which can be identified in the blood film. Evaluation of platelet morphology is made with special attention to size.

Erythrocytes

Mature erythrocytes in avian blood films are elliptical and have an elliptical, centrally positioned nucleus. Erythrocytes of birds should be evaluated on the basis of size, shape, color, nuclear morphology and position, and presence of cellular inclusions. The immature erythrocytes

begin as nucleated spheres that turn into flattened ellipsoids during the final stages of maturation. Unlike mammalian erythrocytes, erythrocytes of birds retain their nuclei where the nuclear chromatin is uniformly clumped and becomes increasingly condensed with age. In Wright-stained blood films, the nucleus stains purple, whereas the cytoplasm stains orange-pink with a uniform texture.

In most species of birds, erythrocyte shape is relatively uniform. The long axis of the nucleus is parallel to the long axis of the cell. Atypical erythrocytes may vary in both size and shape and semiquantitative estimates of these changes can be made from evaluation of monolayer areas of the blood film. The presence of macrocytes or microcytes should be noted during assessment of the blood film. According to Weiss (1984), the degree of variation in the size of erythrocytes (anisocytosis) can be scored from 1+ to 4+ based on the number of variable-sized erythrocytes in a monolayer field. Herbert, et al. (1989) noted erythrocyte subpopulations occur in some species of birds (i.e. ducks), in which larger erythrocytes most likely represent those most recently released from the hematopoietic tissue and smaller cells most likely represent the older, aging cells. A slight variation in the size of erythrocytes (1+ anisocytosis) is normal for birds. A greater degree of anisocytosis, however, usually is observed in birds with a regenerative anemia and is associated with polychromasia. Microcytic, hypochromic, nonregenerative anemia is often associated with chronic inflammatory diseases in birds, especially those with an infectious aetiology as noted by Tell, Ferrell, and Gibbons (2004).

Minor deviations from the normal shape of avian erythrocytes (1+ poikilocytosis) are considered to be normal in the peripheral blood of birds, but marked poikilocytosis may indicate erythrocytic dysgenesis. For example, round erythrocytes with oval nuclei occasionally are found in the blood films of anemic birds and suggest a dysmaturation of the cell cytoplasm and nucleus, which may be a result of accelerated erythropoiesis.

Variations in erythrocyte color include polychromasia and hypochromasia. Polychromatophilic erythrocytes are similar in size to mature erythrocytes and appear as reticulocytes when stained with vital stains, such as new methylene blue. The cytoplasm appears weakly basophilic, and the nucleus is less condensed than the nucleus of mature erythrocytes. Polychromatophilic erythrocytes occur in low numbers (usually <5% of erythrocytes) in the peripheral blood of most normal birds. The degree of polychromasia can be graded based on the number of polychromatic erythrocytes in a monolayer field. Hypochromatic erythrocytes are abnormally pale in color compared with mature erythrocytes, and they have an area of cytoplasmic pallor that is greater than half the cytoplasmic volume. They also may have cytoplasmic vacuoles and round, pyknotic nuclei. The degree of hypochromasia can also be estimated based on the number of hypochromatic erythrocytes in a monolayer field. The presence of many hypochromatic erythrocytes (i.e., 2+ hypochromasia or greater) indicates an erythrocyte disorder such as iron deficiency.

The nucleus may vary in its location within the erythrocyte, and may contain indentions, protrusions, or constrictions. Gómez-Meda, et al. (2006) and Wolf, Niehaus-Rolf, and Luepke (2002) noted that the presence of micronuclei and nuclear budding are potential indices of environmental genotoxic exposure. Lucas and Jamroz (1961) reported chromophobic streaking is indicative of chromatolysis and that achromic bands indicating nuclear fracture with displacement of the fragments may be present as well. Mitotic activity is occasionally noted in blood films and is suggestive of a marked regenerative response or erythrocytic dyscrasia. Binucleate erythrocytes, when present in large numbers along with other features of red blood cell dyscrasia are suggestive of neoplastic, viral, or genetic disease as reported by Romagnano (1994). Anucleated erythrocytes (erythroplastids) or the presence of cytoplasmic fragments are occasionally noted in normal avian blood films.

Leukocytes

The granulocytes in the blood film of birds vary in appearance but can be classified as heterophils, eosinophils, and basophils. The avian heterophil is functionally equivalent to the mammalian neutrophil, and is the most abundant granulocyte of many species of birds. The nucleus (typically partially hidden by the cytoplasmic granules) of the mature heterophil is lobed (two to three lobes), with coarse, clumped, purple-staining chromatin. The cytoplasm of normal mature heterophils appears colorless and contains granules that stain an eosinophilic color (dark orange to brown red) with Romanowsky stains. Typically, the cytoplasmic granules appear elongate (rod or spiculate shaped), but may appear oval to round as well depending upon the species.

The nucleus of the avian eosinophil is lobed and usually stains darker than the nucleus of a heterophil, and the cytoplasm stains clear blue in contrast to the colorless cytoplasm of normal mature heterophils. The cytoplasmic granules are strongly eosinophilic in appearance and tend to stain more intensely compared to the granules of the heterophil on the same stained blood film. It should be noted that some staining techniques, such as Diff Quik, may result in variations in the staining quality among these cells in the various species of birds making cell identification a challenge. The granules are typically round, although the granules of some avian species may be oval or elongate.

The basophils of birds contain deeply metachromic granules that often obscure the nucleus. The nucleus is usually non-lobed, causing avian basophils to resemble mammalian mast cells.

Monocytes are generally the largest leukocytes in peripheral blood films of birds. The monocyte nucleus varies in shape (round or oval to lobed) and the moderately abundant cytoplasm is typically light blue-gray and may be vacuolated. The granules, when present, are very fine and appear azurophilic in Romanowsky stained preparations.

The appearances of lymphocytes in the blood films of birds resemble those of mammals. Their appearance may vary depending upon the species, lymphocyte type, and degree of activation. Lymphocytes vary in size, color of cytoplasm (light to dark blue), and degree of nuclear chromatin condensation. Variability depends on the degree of antigenic stimulation and type of lymphocyte. The size of lymphocytes ranges from the size of a thrombocyte to the size a heterophil. The small lymphocytes are considered to be the inactive forms. Reactive lymphocytes have a slightly more abundant cytoplasm that stains basophilic and nuclei that have clefts or are irregular in shape. According to Weiser (2012), these cells are considered to be B cells involved in immunoglobulin production. Variably sized large lymphocytes that have an increased amount of light-blue cytoplasm and azurophilic granules are considered to be T cells or natural killer cells as reported by Weiser and Thrall (2004).

Acquired changes in leukocyte morphology

In general, the leukocyte morphology is a reliable indication of disease. In avian blood films, the presence of immature cells and toxic heterophils are more reliable criteria for infectious diseases than are total leukocyte and differential counts. Immature avian heterophils have increased cytoplasmic basophilia, nonsegmented nuclei, and immature cytoplasmic granules when compared to normal mature heterophils. Toxic changes are subjectively quantified as to the number of toxic cells and severity of toxicity present, as in mammalian hematology and indicated by Weiss (1984). Toxic change in avian heterophils is a term referring to morphologic changes associated with inflammatory diseases that alter bone marrow production of these types of cells. In response to the inflammatory disease, an acceleration of heterophil production occurs, resulting in the production and release of early stage heterophils with retained organelles such as ribosomes. Retention of these organelles results in cytoplasmic basophilia and the presence of cytoplasmic vacuolation. Döhle bodies may also be present. Döhle bodies are composed of aggregates of endoplasmic reticulum and appear as gray-blue cytoplasmic inclusions.

In mammals and presumably true in birds as well, eosinophils are particularly numerous in the peripheral blood when antigens are continually being released, as occurs in parasitic disease (especially those involving larvae of helminths) and allergic reactions (especially those associated with mast cell and basophil degranulation). In general, the presence of an eosinophilia is suggestive of one of these processes.

Thrombocytes

The thrombocyte is a nucleated cell that represents the second most numerous cell type (after erythrocytes) in blood films of birds. Thrombocytes are typically small, round to oval cells (smaller than erythrocytes) with a round to oval nucleus that contains densely clumped chromatin. They tend to have a high nucleus to cytoplasm ratio (N:C). The appearance of the cytoplasm is an important feature used to differentiate thrombocytes from small, mature lymphocytes. The cytoplasm of normal mature thrombocytes is colorless to pale gray and may be reticulated in appearance compared to the homogenously blue cytoplasm of the lymphocyte in the same Romanowsky-stained blood film. Thrombocytes frequently contain one or more distinct eosinophilic (specific) granules located in one area of the cytoplasm. Thrombocytes are frequently found in clumps on the blood film.

Blood parasites of birds

Parasites in the genera *Haemoproteus*, *Plasmodium*, and *Leukocytozoon* and microfilaria of filarial nematodes are commonly found in avian blood films. Microfilarial nematodes are typically found between the cells. Examination of the whole blood film at low magnification is often necessary to reveal blood parasites, such as microfilaria. *Haemoproteus*, *Plasmodium*, and *Leukocytozoon* produce merozoites that invade erythrocytes and their gametocytes are found within the erythrocyte. Most of the species of *Haemoproteus* and *Leukocytozoon* that infect birds are host specific. Peirce, et al. (2004) reported that many species of *Plasmodium* can infect a wide range of hosts. Parasites of the genus *Plasmodium* can be pathogenic and are responsible for malaria. Castro et al. (2011) indicated that certain species of birds, such as canaries, penguins, ducks, pigeons, raptors, and domestic poultry, are highly susceptible to avian malaria, while other species of birds appear to be asymptomatic carriers of the parasite and do not develop the clinical disease.

Michot, Garvin, and Weidner (1995) and Evans and Otter (1998) reported that in general, the presence of most blood parasites in wild birds has no effect on the health of the bird, although combined infections with *Haemoproteus* and *Leukocytozoon* can produce a fatal anemia. Birds may be infected with a single blood parasite, or may have mixed infections based on examination of stained blood films.

Haemoproteus only appears in the peripheral blood of birds in the gametocyte stage. The appearance of the gametocyte is variable and may range from small developing ring forms to the elongate crescent-shaped mature gametocytes that partially encircle the erythrocyte nucleus to form the characteristic “halter-shape” as originally described by Soulsby (1982). Mature gametocytes typically

occupy greater than one-half of the cytoplasmic volume of the host erythrocyte and cause minimal displacement of the host cell nucleus: the nucleus is never pushed to the cell margin. *Haemoproteus* gametocytes contain refractile, yellow to brown to black pigment granules representing iron pigment deposited as a result of hemoglobin utilization. Erythrocytes parasitized by *Haemoproteus* are larger than normal erythrocytes, which likely causes the cells to become fragile.

Key features used to differentiate *Plasmodium* from *Haemoproteus* are the presence of schizogony in the peripheral blood, parasite stages within thrombocytes and leukocytes, and gametocytes (which also contain refractile pigment granules) that cause marked displacement of the erythrocyte nucleus. According to Soulsby (1982), identification of the *Plasmodium* species is dependent upon the location and appearance of the schizonts, the number of merozoites present within the schizonts, and gametocytes.

Leukocytozoon only appears in the peripheral blood of birds in the gametocyte stage that grossly distorts the host cell (presumed to be an immature erythrocyte). Bennet and Pierce (1992) indicated that the macrogametocyte appears as a parasite inclusion that occupies 77% of the area of the host cell-parasite complex and the microgametocytes are similar in morphology, but are usually 5%–10% smaller. The remainder of the life cycle occurs in the insect vector following ingestion of blood containing the gametes as indicated by Gardiner, Fayer, and Dubey (1988).

Aegyptianella is a piroplasm that can affect several species of birds, usually those originating in tropical or subtropical climates. *Aegyptianella* appears as a minute parasite lacking pigment granules located within erythrocytes in blood films. Soulsby (1982) indicated that three forms of this parasite can occur within the erythrocyte. These include a small (<1 µm), round, basophilic intracytoplasmic, anaplasma-like inclusion; a *Babesia*-like round to piriform-shaped inclusion with pale blue cytoplasm and chromatin body at one pole; and a larger (2–4 µm) round to elliptical inclusion.

Atoxoplasma is a coccidian parasite often found in passerine birds. Atoxoplasmosis is identified by the presence of the characteristic sporozoites within lymphocytes in peripheral blood films. Affected lymphocytes contain small (3–5 µm), pale, round to oval eosinophilic intracytoplasmic inclusions or sporozoites that indent the host cell nucleus, resulting in a characteristic crescent shape in Romanowsky-stained preparations.

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