Clinical Technique: Amphibian Hematology: A Practitioner's Guide

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Abstract

Amphibian hematology is challenging because of a combination of several factors including small patient size, few venipuncture sites, lack of normative data, and basic variability of the amphibian leukocyte and erythrocyte counts. This variability in amphibian red blood cell and white blood cell counts is based on a number of extrinsic (e.g., temperature, diet, season, light cycle) and intrinsic (e.g., species, gender, life style) factors. If possible, to best guide amphibian hematological interpretation, a conspecific, same gender animal can be sampled for comparison to dispel extrinsic and intrinsic variability. However, the collection of hematological measurements in the single amphibian patient can still provide useful clinical information to guide therapy of even the most diminutive amphibian patient. Therefore, the following brief guidelines are presented in an attempt to guide the clinical practitioner as to collection and interpretive techniques, which can easily be adapted to clinical practice for these fragile jewels of nature. Equipment necessary for venipuncture, venipuncture sites, a venipuncture technique, a technique for determination of an estimated white blood cell count and differential, and a guide for differentiation of leukocytes of the amphibian are given. This guide should by no means supplant a thorough review of the literature or consultation with a clinical pathologist, but will provide general rules of thumb for quick interpretation. Excellent reviews of sampling and complete blood count interpretation are listed in the references.

Key words: **amphibian; hematology; blood collection; venipuncture; clinical pathology**

When collecting blood from amphibian species, one should use appropriate materials for venipuncture, proper handling of the animal, and more commonly, sedation or anesthesia. Use suitable restraint including talc-free, distilled water-washed examination gloves and a deionized water-washed surface; give due consideration to sedation or light anesthesia to give the best chance of successful sample collection and the least chance of harming the amphibian. At the minimum, be sure to record the following information with any amphibian hematology sample: time, date and site of collection; patient species, gender, age, and life stage; and season, diet, and light cycle.

Recommended materials for amphibian blood sampling and blood smear preparation are given in Table 1 and Figure 1. Syringes may be precoated by drawing a small amount of heparin into the syringe and then expelling any excess if a difficult or slow blood collec-

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Table 1. Equipment necessary for amphibian hematological sampling

Equipment	Manufacturer	Comments
Accusure Insulin Syringe 0.3-0.5 mL	Qualitest Pharmaceuticals, Inc., Huntsville, AL	Zero volume hub, attached needle 28 gauge, 1/2''
Selected micro cover glasses	VWR Scientific Inc., Westchester, PA	"Cover slips" for blood smear prep
Gold Seal Rite-On Microscope Slides	Erie Scientific, Portsmouth, NH	1.0-mm thickness
Dog nail trimmers, guillotine style	Resco, Walled Lake, MI	Removal of hub/needle of zero volume attached needle syringes; conserves sample volume
Heparinized hematocrit tubes	Drummond Scientific Company, Broomall, PA	PCV and total solids determination; may be used to create small drop size to facilitate amphibian blood smears
Lithium heparin microtainers	Becton-Dickinson, Franklin Lakes, NJ	May be used to store hematologic samples

tion procedure is expected. Additionally, if sepsis is a concern, consider reserving one drop of blood without anticoagulant for placement onto a mini-tip culturette or directly into an enrichment broth, which then should be incubated at enclosure temperature. When collecting blood for culture, aseptic technique, which includes adequate patient preparation and strict surgical preparation of the skin to avoid culture contamination, is necessary. Contacting the bacteriological laboratory for appropriate materials, such as pediatric blood culture vials, and instructions for sample collection and storage should occur before the procedure for best diagnostic results. Although the collection of samples before antibiotic administration is preferred in a patient where sepsis is a concern, the collection of samples after antibiotic administration is not contraindicated.

Methods of blood collection vary based on the species. Most commonly used venipuncture sites include the ventral caudal tail vein, the ventral abdominal vein, and the heart. Peripheral venipuncture may not require chemical restraint and may be performed in a manner similar to that used on the tail vein of bovids and as in reptilian species for the ventral abdominal vein. For the abdominal vein or cardiopuncture, once the animal is positioned appropriately, it is best to visualize the venipuncture site. For the abdominal vein, visualization may be aided by a bright, cool fiber-optic light source to transilluminate the vein from the side or behind or by ultrasound guidance. The ventral abdominal vein may be visualized without additional aids as it courses down the ventral midline in amphibians with minimally pigmented, translucent skin. The cardiac impulse can be visualized in some species without additional aids. However, the dense skin pigmentation of many species necessitates the use of a Doppler flow probe (Fig 2), ultrasound, or cool light transillumination to confirm the blood flow in the heart or in other vessels before venipuncture. As in other species, cardiac puncture is not without risk and should be performed only in an anesthetized animal with blood aspiration from the ventricle with a small-gauge needle (Fig 3).

A small-gauge needle limits trauma to the amphibian and will not damage blood cells as long as minimal aspiration pressure is applied. Use of an insulin syringe with a zero volume hub also maximizes recovery of the sample; however, the needle must then be removed for

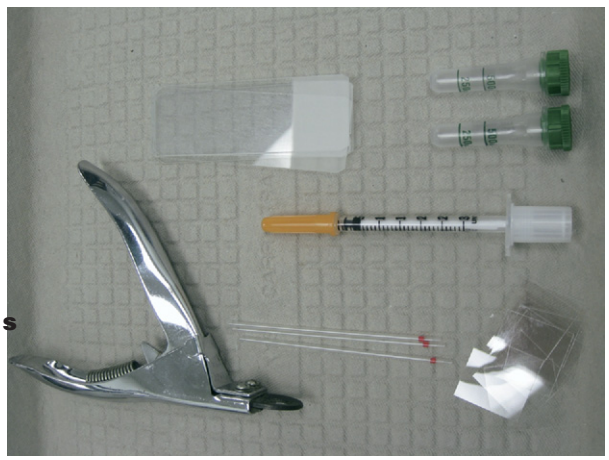

Figure 1. Basic equipment for amphibian hematological sampling. Pictured: Guillotine canine nail trimmers, 0.3-mL insulin syringe with zero hub needle attached, slides, cover slips, heparinized PCV tubes, and lithium heparin microtainers. Additional materials for restraint of the amphibian are necessary (talc-free examination gloves washed with distilled or deionized water, large dog mask for use as induction chamber for isoflurane anesthesia, nonbleached paper toweling or surface which has been moistened with distilled or deionized water).

Figure 2. Placement of a Doppler flow probe over the heart of a Malaysian Horned Frog (*Megophrys nasuta*) to verify appropriate venipuncture site.

sample processing. For needle removal, pull the sample to the back of the syringe and remove the needle and hub with clean, sharp, guillotine-style dog nail trimmers. This allows sample handling without having to expel the blood through the small needle. Blood may be used for fresh smears, culture, packed cell volume, and total solids determination. Blood smears may be made with a slide or cover slip technique. The use of heparized hematocrit tubes may facilitate making the small drops necessary for good blood smears when evaluating amphibian blood. The remainder can then be placed in the lithium heparin microtainer for separation of plasma and determination of blood chemistries. Normal amphibian plasma varies with the species and may be light blue, green, or orange. In the amphibian, green plasma may or may not be due to biliverdin elevations, and yellow or orange coloration may be due to carotenoids or bilirubin elevations. Blue plasma has been found in apparently healthy Japanese giant salamanders (*Andrias japonicus*). The clinician should exercise caution in the overinterpretation of the plasma coloration in amphibian species as the cause for coloration of serum or plasma, be it based disease or completely normal, only has not been investigated in most species.[1]

For the leukocyte differential of amphibian blood cells, a tabular (Table 2) and pictographic guide (Figs 4-10) are given. Blood cells shown were obtained from Malaysian Horned Frogs (*Megophrys nasuta*) via cardiac venipuncture. Amphibian white blood cells tend to vary considerably in morphology and staining characteristics based on species. Therefore, these cells are presented only as a cursory guide. One should consult additional texts for best interpretation of amphibian leukocytes.[1-3]

Procedural Tip I

Calculation of the Appropriate Volume of Blood to be Sampled from an Amphibian

The guidelines below calculate a minimum safe blood draw. Conservative measures are used at each step to safeguard patient safety.

- Blood volume varies from 7% to 10% of body weight in the terrestrial amphibian and 13% to 25% of body weight in the aquatic amphibian.
  (Body weight, g)(0.07) = Minimal total blood volume (TBV)
  In the aquatic amphibian, the multiplication factor may be increased to 10% or 0.1.
- Minimal safe blood draw (SBD) = 5% to 10% of TBV
  TBV (0.05) = SBD
  In the healthy amphibian, SBD may be doubled.
- For a 35-g sick frog, SBD:
  35(0.07)(0.05) = 0.12 mL

Figure 3. Blood collection via cardiac venipuncture of a Malaysian Horned Frog (*Megophrys nasuta*). This frog has been anesthetized with isoflurane administered via face mask.

Table 2. Differentiation of common amphibian blood cells[1,2]

Cell Name*	Relative Cell Size, Shape, and Percentage	Nucleus	Cytoplasm/Granules
Neutrophil	Predominant inflammatory cell in some species; visually similar to mammalian cell	Multilobed nucleus	Colorless cytoplasm, large irregular granules with slight eosinophilia or only slight stain uptake
Heterophil (small eosinophilic granule leukocyte)	30 to 32 μm; larger than that of mammals; 1% to 33% of leukocytes	Round, multilobed nucleus	Colorless cytoplasm, distinct eosinophilic, rod-shaped, smaller than eosinophil granules; may phagocytize bacteria
Eosinophil (large eosinophilic granule leukocyte)	Same size or larger than heterophil; 0% to 15% of leukocytes	Smooth, less lobed nucleus than heterophil	Small to medium, oval to round eosinophilic granules of variable size
Basophil	Size variable; $<$1% of leukocytes of most species; may predominate in some	Nonsegmented, unlobed, slightly eccentric, often obscured by granules	Basophilic granules round to oval; often degranulated by Diff-Quik stain
Monocyte/Azurophil	Similar in appearance to mammalian cells; usually larger than granulocytes but varies from smaller to larger; 0% to 20% of leukocytes	Round, kidney, or horseshoe shaped	Pseudopodia may be present, light blue-gray, abundant cytoplasm foamy or vacuolated; occasional small, irregular-shaped azurophilic granules (azurophils); may phagocytize other cells
Small lymphocyte (large lymphocyte)	Similar in appearance to mammalian cells; $\sim$50% of differential in many species; smallest leukocyte (small lymphocyte) more numerous than large lymphocyte; 1/2 size of erythrocyte or granulocyte	Usually round but occasionally cleft or bilobed, nuclear chromatin dense, clumped	Scant, small amount, pale blue to deep blue gray; less cytoplasm with more intense color than thrombocyte
Thrombocyte	Oval to spindle shape; cells tend to aggregate	Dense to round oval	Abundant colorless/pale gray
Erythrocyte	Larger than that of mammals; oval cell shape	Usually nucleated, nucleus basophilic with bulge, irregular nuclear margin	Deep eosinophilic (red to pink)

**Cell name is based on amphibian cell staining characteristics and does not imply specific cell function or other similarity to mammal or other vertebrate cells.*

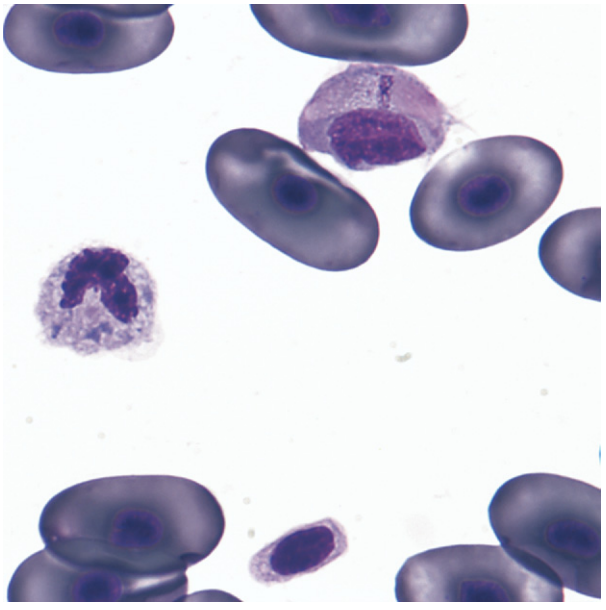

Figure 4. Blood smear from the terrestrial Malaysian Horned Frog (*Megophrys nasuta*) stained with Diff-Quik shown at 1000× magnification. The leukocyte to the left of the image is a neutrophil with Döhle bodies, the leukocyte on top is a large lymphocyte with an intracellular hemoparasite, and the small cell on the bottom is a thrombocyte.

From a minute blood sample (0.1 mL), one can examine for hemoparasites, perform an estimated white blood cell count and a blood culture, and obtain a packed cell volume and total solids determination with a microhematocrit tube. Obtaining an accurate white blood cell count with the help of a hemocytometer requires only about 0.02 mL if the blood is appropriately diluted with diluent/stain.

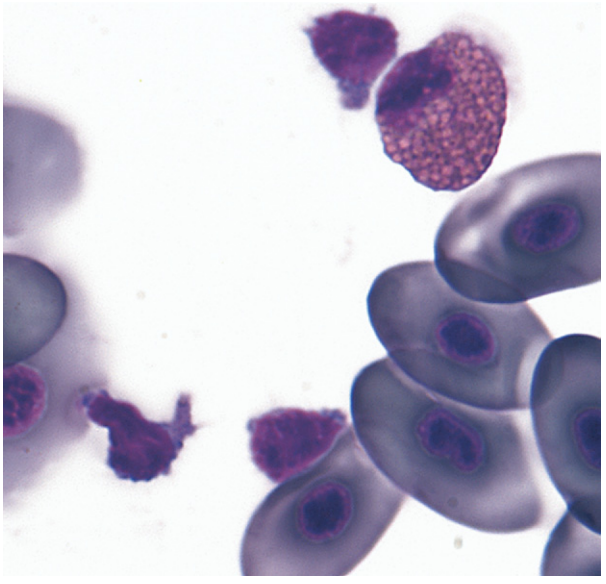

Figure 5. Blood smear from the terrestrial Malaysian Horned Frog (*Megophrys nasuta*) stained with Diff-Quik shown at 1000× magnification. The eosinophil is the large cell with round cytoplasmic granules. The remaining three leukocytes are small lymphocytes.

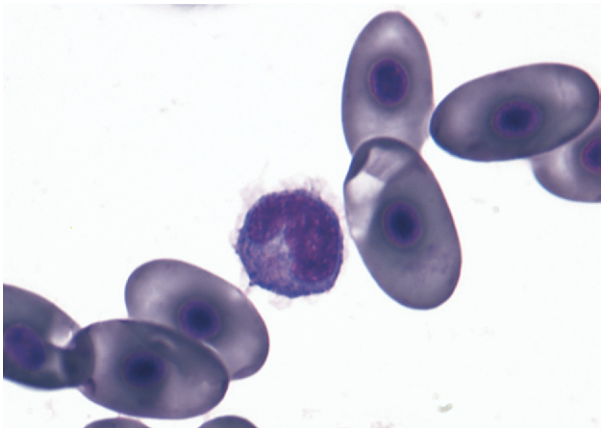

Figure 6. Blood smear from the terrestrial Malaysian Horned Frog (*Megophrys nasuta*) stained with Diff-Quik shown at 1000× magnification. The leukocyte shown is an azurophilic monocyte.

Procedural Tip II

Estimation of the Total White Blood Cell Count and Determination of the Differential Cell Count in an Amphibian

- In the cell monolayer, count the number of white blood cells in 12 high powered (400×) fields.

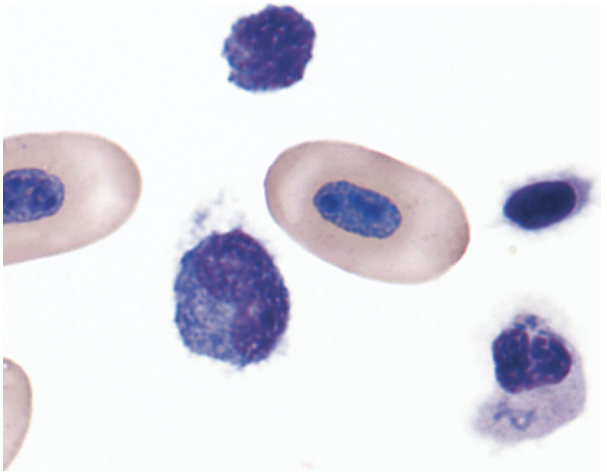

Figure 7. Blood smear from the terrestrial Malaysian Horned Frog (*Megophrys nasuta*) stained with modified Wright's stain and shown at 1000× magnification. From the top proceeding clockwise, a small lymphocyte, a thrombocyte, a neutrophil, and azurophilic monocyte are shown.

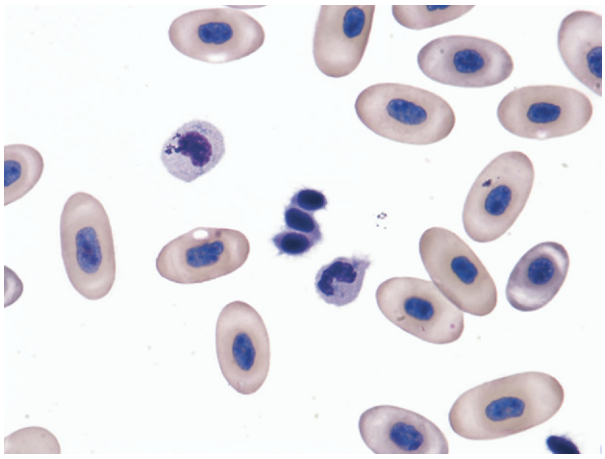

Figure 8. Blood smear from the terrestrial Malaysian Horned Frog (*Megophrys nasuta*) stained with a modified Wright's stain and shown at 1000× magnification. From the top, leukocytes shown are a monocyte, 3 aggregated thrombocytes, and a neutrophil.

- Subtract the highest and lowest number fields, then divide the remaining total by 10 to obtain X.
- Multiply X by 1000. The final number is an estimate of the amphibian total white blood cell count = LC.
- To obtain a cell differential, count 100 white blood cells to obtain a percentage of each white blood cell type (see Table 2). Multiply each cell's percent by LC to give an estimated leukocyte count per cell.

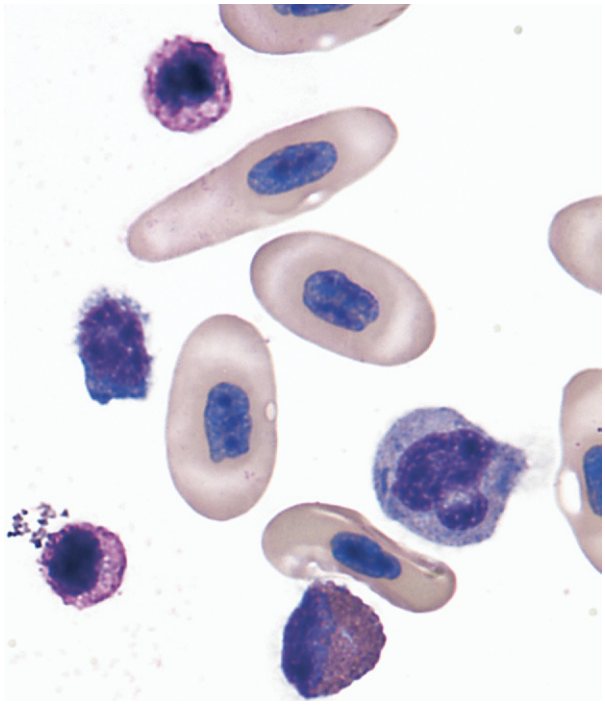

Figure 9. Blood smear from the terrestrial Malaysian Horned Frog (*Megophrys nasuta*) stained with a modified Wright's stain and shown at 1000× magnification. From the top, a basophil, a small lymphocyte, a monocyte, another basophil, and an eosinophil can be seen.

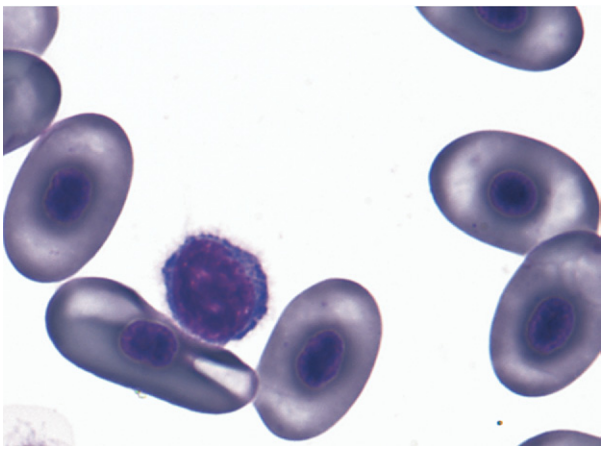

Figure 10. Blood smear from the terrestrial Malaysian Horned Frog (*Megophrys nasuta*) stained with Diff-Quik (Fisher Scientific, Pittsburg, PA USA) shown at 1000× magnification. The central leukocyte is a large lymphocyte.

Summary

Amphibian hematology is still in its infancy, and sample collection and interpretation may be daunting to the general practitioner. This basic summary reference guide was prepared to facilitate interpretation of the amphibian complete blood count for the veterinary clinician. By making amphibian hematology interpretation more uniform and accessible, it is hoped that the standard of amphibian medicine will improve and that amphibian hematology will no longer be regarded as a rare diagnostic procedure in these patients. Hopefully, both amphibians and mankind will benefit from this advancement.

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