Avian and Reptile Hematology and Sample Submission: What is the Normal?

Kendal Harr DVM, DACVP
URIKA, LLC
Mukilteo, WA

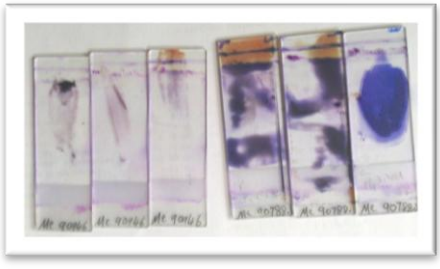
General sample collection

Collection of blood volume equivalent to 1% of a bird's body weight and 0.5% of a reptile's body weight is usually not associated with any adverse effects. Debilitation and dehydration should be considered. For hematology samples from all mammals and most bird and reptile species (not chelonian/turtles or ratites and corvids) appropriate volume EDTA tubes (likely microtainer tubes) are preferred if possible. Inappropriate blood to anticoagulant ratios will cause lysis. Additionally, bird blood will lyse during transport over 12 hours in duration regardless of anticoagulant used.

Plasma chemistry using lithium heparin is preferred in nonmammalian species to insure that there is a fluid component to analyze and to extend sample volume in small animals. Serum samples will occasionally form a hard gel due to fibrin that cannot be analyzed and results in an unusable sample. In the United States, many exotics practitioners perform venipuncture using a needle that is heparinized in house with injectable sodium heparin. While this is not always necessary, it may be done if there is significant concern about obtaining a high quality sample. If most of the heparin is expelled, it will minimally affect the sample. However, this can vary between practitioners and samples. Any droplets remaining may cause dilutional effects as well as interfere with some analytical tests, such as sodium and albumin.

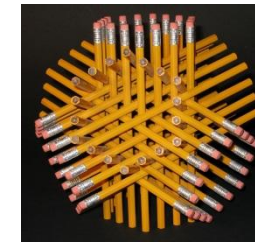
Hemolysis changes results. Many practitioners use tuberculin or insulin syringes that do not have detachable needles. These needles can easily be cut from the syringe using a pair of large veterinary nail clippers to prevent the inevitable hemolysis which occurs when blood is squeezed back through a tiny needle.

Sample preparation



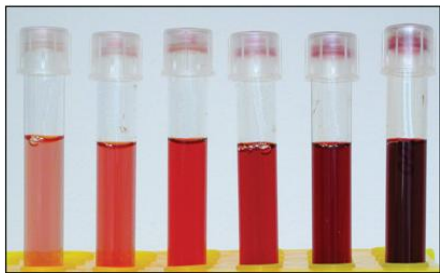
Smears, either cover slip preparations or pull or push preps should be gently made immediately (within half an hour of sampling) to insure intact cells for cell counts and morphologic evaluation. Cover slip preparations are more gentle and may provide a solution if preparations are frequently lysed. A small drop of blood is placed between two coverslips which are laid caddy corner to each other. They are then slid laterally to form two preparations in which the majority of the preparation can be analyzed. Coverslips may be problematic in that they are difficult to appropriately label and frequently break. Cover slips can be inverted and glued to a slide with unstained blood facing up. This makes them more sturdy for transport and allows appropriate labeling with date and name at the frosted edge of the slide.

Note that it is important to make cytologic or hematologic smears such that the sample is in the middle of the slide. As you can see at left, the edges of the central 3 bone marrow and cytology preparations are not stained. This is typical of many of the automated stainers used in diagnostic laboratories. Therefore, samples at the edge of the slide will not be evaluated and this is one way to result in an interpretation of nondiagnostic. When the feathered edge of a CBC smear is there, the slide is not useable.



When labeling slides, most inks, except expensive specially designed laboratory pens, will wash off in the methanol fixative of the stain, leaving the slide unlabeled and, therefore, unusable. Most laboratories will label your slide with a number for you to prevent this. However, any information that you wish to convey to the pathologist may not be received. I highly recommend slide labeling with pencil. Using plain old pencil results in permanent labeling of slide and is cheap and easy.

Changes in temperature during transport are the enemy. Increased temperature can increase cell metabolism changing some analyte values and cells can lyse, causing hemolysis and more error. Of course freezing will also cause cell lysis and whole blood should only be frozen for analyses where cell morphology doesn't matter such as PCR are toxicological analysis.



Avian plasma samples are frequently yellow due to carotenoid pigments, not bilirubin. Pink or red plasma is indicative of hemolysis. Hemolysis may cause increases in LDH, AST, potassium, and bile acids (colorimetric). Extended transport time or increased temperature during transport can cause variable potassium, calcium, and phosphorous values, decreased glucose concentration, and increased LDH, AST, and potentially protein concentration due to diffusion/transport across the RBC membrane. I would not recommend running any of the hemolytic tubes after the first one at right as significant changes in analytes may lead to misdiagnosis. What you see go out the clinic door and what we see when it enters the laboratory may be two very different things, especially in today's world of centralized laboratories and occasional extended transport. Biochemical samples should always be separated within 30 minutes to prevent artifact that may cause misdiagnosis. Lipemia is rarely observed in avian samples, however, obese Amazons and other birds may produce lipemic plasma. This may be indicative of underlying metabolic disease such as hypothyroidism or diabetes mellitus.

Many veterinarians are not adequately equipped to manage exotic species and receive minimal training in appropriate handling of blood. Discussion with the practitioner about appropriate materials, venipuncture technique, and sample handling can significantly improve the quality of the sample and, therefore, the result.

ASVCP hematology guidelines

Regardless of species, no clotted samples should be run for quantitative hematologic analysis (CBC) according to the ASVCP guidelines as these samples give imprecise and variably inaccurate results. I recommend all practices send 5 known clotted samples to their reference laboratory or potentially as a practice through their in-house laboratory to make sure that this quality assurance policy is being followed. The results may surprise and concern you. . If your lab is either missing or not reporting clotted samples, they are not functioning within guidelines and are likely providing you with inaccurate results a percentage of the time. You WANT to get a phone call telling you that your sample is clotted

The following are directions that your reference lab should be following

"Anticoagulated samples for hematology that are found to have macroclots that can be found upon visual inspection will produce variably erroneous results. The clinic should be contacted either in writing or by phone and informed that the sample will produce erroneous results. Because the varying degree of inaccuracy cannot be predicted, clotted samples are unsuitable for analysis and it is not recommended that these samples be analyzed. If samples of questionable or substandard quality are analyzed, any procedures and possible inaccuracies should be documented in writing by the laboratory. Additionally, any possibly inaccurate results should have easily seen comments on the report to the clinician that clearly state those values may be inaccurate and misleading."

I. Blood films made in the clinic should not be refrigerated and should be protected from condensation and freezing during transport to the laboratory.
    a. (A dry piece of paper towel or other absorbant material inserted in the packaging may be helpful.)
II. Equipment (e.g. hemacytometers, weighted hemacytometer cover slips, hand tallies, calibrated pipettes, differential cell counters) used for hematology procedures should be in good working order.
III. Routine monitoring and regular maintenance of each piece of equipment (e.g. annual calibration of pipettes and balances) should be performed and documented. Records of maintenance, and malfunction and repairs should be kept.
IV. Manual WBC counts using a hemacytometer are imprecise and have coefficients of variation ranging from 20-40% (Schalm, Harr et al, 2005); therefore, quality control implementation and statistical analysis may yield significance or a lack of significance that is not relevant to daily operation. Method validation studies for shark species conducted by J. Arnold (2005) showed coefficients of variation comparable to manual hematology for human WBC counts as reported in the B-D product insert for Unopette 365855 when the sample was processed within 5 hours of collection.
V. Currently, commercially prepared control materials are not available for non-mammalian blood cell counts. Procedural controls include:
    a. Duplicate dilutions from a patient specimen, performed within the acceptable time limits for sample stability.
    b. WBC Estimate from Blood Smear. Each institution should document a protocol to achieve a reliable method for evaluating the accuracy of the hemacytometer counts. Estimated total WBC values may be difficult due to the similar morphology of lymphocytes and thrombocytes when viewed at lower magnifications typically used for WBC estimates of mammalian cells.
VI. Proficiency testing for technologists should be documented annually, or more frequently as determined by the institution.
    a. Testing should include comparison counts from the same blood sample for total cell counts and for leukocyte differentials.

1. Sample selection should be representative of patient population (avian, reptile, teleost, elasmobranch, etc.).
2. Between technologists, hemacytometer counts should agree to within 15% and differential percentage results for each cell type should agree to within 95% confidence interval.

VII. Direct Cell Count Method—Thrombocyte/Lymphocyte Error. It may be difficult to differentiate between thrombocytes and lymphocytes in the hemacytometer or on stained smears for that matter for newly trained technologists, or for experienced technologists when counting some animal species. Personnel education, proficiency testing, and quality controls should be performed regularly

Urinalysis – All species

Some of the worst samples that I have had to try to deal with are urine samples from any species. Urine is caustic and cells, especially neoplastic cells, degrade very quickly. Significant pH change may occur, due to bacterial overgrowth, that will alter crystal formation. Crystals are best evaluated on fresh wet mount quickly after sampling with no stain added. Cytologic sample should be placed in purple top tubes or urine collection tubes to prevent bacterial overgrowth. Cytologic evaluation should use stain, my preferred being Diff Quik. I do not advocate Sedi-Stain as it frequently results in misdiagnosis. Preparation of urine sediment to smear on slide will preserved the sample for shipment and enable identification of neoplastic cells. To prepare urine in clinic, centrifuge urine in the clinic and make two pull-prep smears of the sediment. This will preserve cells for approximately one week.

Sampling

Identification of the urine collection method is important when interpreting the presence and concentration of potential contaminants including blood and bacteria. The submitter should clearly state the method by which the urine was obtained, such as free flow (midstream, early, or late), catheterization, cystocentesis, or from the floor or metabolism cage. Clear specimen containers can be used to facilitate gross examination if urine will be examined within 30 minutes. However, if urinalysis will be delayed, urine should be protected from exposure to UV light to prevent degradation of urine constituents (eg, bilirubin). Lids should be secure to prevent evaporation and/or volatilization of urine constituents (eg, ketones).

Transport and storage

Optimally, urine should be examined within 30 minutes of collection. If immediate examination is not possible, urine should be stored at refrigerated temperatures to minimize changes in urine physical and chemical make up and to inhibit bacterial growth. Strict recommendations for duration of refrigerated storage cannot be made, because this depends on specific urine components. Storage for a maximum of 24 hours in the refrigerator is generally recommended (Osborne cautiously suggests 6-8 hours), but urine may be stable for shorter or longer periods depending on its initial make up. Chemical constituents that are particularly unstable include bilirubin and glucose, and pH if bacteria are present. Stability of formed elements depends on urine pH and concentration. Crystals may form in vitro during storage at either room temperature or under refrigeration. If crystalluria is a clinical concern, freshly collected urine should be examined immediately. Refrigerated samples should be brought to room temperature prior to analysis. Because urinalysis results may be affected by storage duration and temperature, the time the urine was collected, the time it arrived in the laboratory, and method of storage should be recorded. Alternative methods of preservation are available for stabilization of urine chemistry, inhibition of bacterial growth, and preservation of formed elements. Manufacturer's claims should be followed regarding intended use of particular preservative and duration of storage.

Quantitative microbiologic culture techniques are recommended for determining presence of significant bacteriuria. Urine specimens collected by cystocentesis are preferred, but specimens properly collected by catheterization and free-catch are acceptable if quantitative culturing methods are employed. Urine should be submitted for the microbiological culture prior to urinalysis procedure to avoid contamination of the specimen. Alternatively, a sterile aliquot can be set aside for possible microbiological culture subsequent to urinalysis procedure. Refrigerated urine samples are acceptable for microbiological culture for at least 6 hours and often up to 24 hours. Refrigeration of urine specimens for 24 hours may result in false negative culture results. If bacteriostatic transport media is used, urine samples do not need to be refrigerated.

A thorough review of all preanalytical issues cannot be provided in this space, please consult the QAS guidelines, http://www.asvcp.org/pubs/qas/newQas/index.cfm as well as manufacturer's recommendations regarding specific methods employed.

Coagulation

>90% of error associated with coagulation analysis in human medicine is attributed to preanalytical error. Therefore, coagulation must be mentioned in any sampling discussion. At this time in exotic species medicine it is relatively uncommon, though prothrombin time (PT) may be used in avian species, e.g. hawks for detection of rodenticide intoxication. For this reason, a flow chart for correct sampling procedure for venipuncture has been finalized and is available here http://www.asvcp.org/pubs/pdf/CoagSampGuide.pdf Whole blood should be collected in trisodium citrate anticoagulant in a 9:1 ratio and thoroughly mixed. This typically is accomplished by filling to the indicated mark on the appropriate blood tube. Specimens that do not conform to this dilution should be rejected.16 Citrate volume may need to be adjusted for samples from very anemic and polycythemic animals.17 For tests requiring plasma, the citrated tube is centrifuged and chilled to 2-8ÂºC. Plasma should be separated from blood cells and transferred to a plastic tube (not glass).18,19 Specimen stability at room or refrigerated temperature (2-8ºC) is 4 hours and 24 hours for APTT and PT, respectively. If testing is not performed within these time intervals, samples should be frozen at -20ºC.20 Fresh citrated whole blood used for platelet function or other coagulation analyses should be ideally kept for <1 hour. (Giger, per communication). If samples are mailed into a laboratory for testing versus direct transport, plasma should be placed in a plastic tube then frozen, then be packed on ice and shipped to arrive frozen within 24 hours.

References

Thrall et al. Veterinary Hematology and Clinical Chemistry. 2004. Chapter 2: Sample Collection, Processing, and Analysis of Laboratory Service Options. P. 39-44.

Vap, L. M., Harr, K. E., Arnold, J. E., Freeman, K. P., Getzy, K., Lester, S. and Friedrichs, K. R. 2012. ASVCP quality assurance guidelines: control of preanalytical and analytical factors for hematology for mammalian and nonmammalian species, hemostasis, and crossmatching in veterinary laboratories. Veterinary Clinical Pathology, 41: 8–17

ASVCP Nonmammalian Clinical Pathology Guidelines. http://www.asvcp.org/pubs/qas/newQas/nonmammal.cfm (accessed August, 2012)

ASVCP Clinical Pathology Guideline Index

http://www.asvcp.org/pubs/qas/index.cfm

When guidelines are chosed in pdf they can be search using the find feature in adobe and the html is indexed.