Journal of Avian Medicine and Surgery 22(2):108–113, 2008

Comparison of Hematologic Values in Blood Samples With Lithium Heparin or Dipotassium Ethylenediaminetetraacetic Acid Anticoagulants in Hispaniolan Amazon Parrots (*Amazona ventralis*)

David Sanchez-Migallon Guzman, Lcdo en Vet, Mark A. Mitchell, DVM, MS, PhD, Stephen D. Gaunt, DVM, PhD, Dipl ACVP, Hugues Beaufrère, Dr Vet Med, and Thomas N. Tully, Jr, DVM, MS, Dipl ABVP (Avian) Dipl ECAMS

Abstract: Blood samples were collected from 20 Hispaniolan Amazon parrots (*Amazona ventralis*) and were divided into tubes that contained dipotassium ethylenediaminetetraacetic acid (K_2EDTA) and lithium heparin. Complete blood cell counts were determined in each sample within 2 hours of collection. The level of agreement in results was moderate for plasma protein, packed cell volume (PCV), and leukocyte, monocyte, and lymphocyte counts between the anticoagulants. Plasma protein and PCV values were significantly lower in samples with lithium heparin than in those with K_2EDTA, whereas lymphocyte numbers were significantly higher in lithium heparin samples than in K_2EDTA samples. The level of agreement was good for the other cell types (heterophils, eosinophils, and basophils) when comparing the different anticoagulants. The poor level of agreement between anticoagulants with the increase in thrombocyte clumping in lithium heparin samples indicates that the use of lithium heparin as anticoagulant may affect thrombocyte count. No negative effects on morphology and staining of blood cells were apparent in smears from heparin samples compared with K_2EDTA samples. Within the different values compared, the limits of agreement are small enough to be confident that lithium heparin can be used for routine CBC counts in a clinical setting. The use of the same anticoagulant should be recommended to follow trends within the same patient, especially when considering plasma protein concentration, PCV, and lymphocyte count.

Key words: Anticoagulant, hematology, lithium heparin, dipotassium ethylenediaminetetraacetic acid, avian, Hispaniolan Amazon parrots, *Amazona ventralis*

Introduction

Dipotassium ethylenediaminetetraacetic acid (K_2EDTA) and lithium heparin are 2 of the most commonly used anticoagulants in avian medicine. The mode of action of K_2EDTA is by binding calcium ions, which are essential in the coagulation cascade and for cell-to-cell interaction. Heparin binds to and accelerates the activity of antithrombin III, which inhibits the action of thrombin and other proteases necessary for coagulation.[1]

From the Department of Veterinary Clinical Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803-8410, USA (Guzman, Gaunt, Tully); the Veterinary Teaching Hospital, Ontario Veterinary College, University of Guelph, Ontario N1G 2W1, Canada (Beaufrère); and the Department of Veterinary Clinical Medicine, College of Veterinary Medicine, University of Illinois, 1008 W Hazelwood Dr, Urbana, IL 61802, USA (Mitchell).

In domestic mammals, K_2EDTA is the recommended anticoagulant for storing blood samples for hematologic testing. In many domestic species, heparin alters leukocyte morphology, provides poorer staining quality to the cells, and does not prevent platelet aggregation.[2,3] In birds, K_2EDTA is also generally recommended by some investigators as the anticoagulant of choice for hematologic testing,[4,5] although it was shown to cause progressive hemolysis of the red blood cells (RBCs) in ostrich (*Struthio camelus*), jackdaws (*Corvus monedula*), ravens (*Corvus corax*), black curassows (*Crax alector*), black crowned cranes (*Balearica pavonina*), gray crowned cranes (*Balearica regulorum*), hornbills (*Tockus alboterminatus*), and brush turkeys (*Alectura lathami*).[4] In those species where this occurs, heparin is the preferred anticoagulant.[4,5] Other investigators report a

preference for heparin for both complete blood cell (CBC) counts and plasma biochemical assay[6] or observed that after 24 hours, blood samples anticoagulated with EDTA degenerate more than other types of anticoagulated blood samples.[7]

Only a few controlled studies compared the hematologic values of avian blood samples collected in heparin and EDTA.[1,8–10] In a study that evaluated pigeon blood, the temporal effect of different concentrations of heparin and dipotassium salts on the hematocrit was evaluated. Results showed EDTA to be superior to heparin in producing a constant hematocrit value as time progressed and in the period of time before hemolysis occurred in the blood.[8] In a similar study, also in pigeons, the effects of different anticoagulants in various concentrations on hematocrit, osmolarity, and pH was studied, and heparin was found to be the most suitable anticoagulant.[9] Most recently, a study in macaws (*Ara* species) compared the temporal effects of 3 commonly used anticoagulants. However, the use of heparin-coated syringes in this study limited the ability to fully ascertain the effect of individual coagulants.[1]

Comparable investigations were recently done in reptiles. In studies that evaluated blood samples from green iguanas (*Iguana iguana*)[11] and yellow-blotched map turtles (*Graptemys flavimaculata*),[12] hematologic results from lithium heparin and K_3-EDTA anticoagulated samples differed significantly, whereas, in another study, which evaluated Burmese pythons (*Python molurus bivittatus*), no significant differences were found between the sample results.[1] In Hermann's tortoises (*Testudo hermanni*), K_3EDTA was shown to lower the PCV.[13] Based on results of these studies in reptiles, differences in hematologic results between anticoagulants may be at the order or species level and not at the class level.

The purpose of this study was to measure the level of agreement and determine significant differences in the hematologic values of Hispaniolan Amazon parrots (*Amazona ventralis*) when blood samples were placed in 2 different anticoagulants. We hypothesized that the level of agreement would be good and that results of the CBC count would not differ significantly between anticoagulants.

Materials and Methods

This study was performed according to the regulations established by the Louisiana State University Animal Care and Use Committee. Twenty Hispaniolan Amazon parrots that belonged to a resident colony at Louisiana State University were used for this study. The birds were fed a pelleted diet (Exact Breeding Formula, Kaytee Products Inc, Chilton, WI, USA) and had unlimited access to water. All birds were apparently healthy based on results of physical examination.

For venipuncture, birds were manually restrained, and a 1-ml blood sample was collected from the right jugular vein of each bird with a 25-gauge needle attached to a 3-ml plastic syringe. Blood samples were collected from 2 birds each day over a 10-day period, and the same person collected all blood samples (DSMG). This schedule was followed to minimize the likelihood of the laboratory being rushed to process all of the samples in a single day and to ensure that all samples were processed within 2 hours of collection. Immediately after collection, each sample was separated into 2 tubes: 1 tube contained K_2EDTA, and the second tube contained lithium heparin (Microtainer, Becton Dickinson and Company, Franklin Lakes, NJ, USA). The order of sample placement into the anticoagulant tubes was alternated between birds. Blood samples were stored at room temperature until processed within 2 hours of collection by the clinical pathology laboratory at the Louisiana State University School of Veterinary Medicine. A single technician who was blinded to animal number reviewed all samples. For each sample, packed cell volume (PCV), plasma protein concentration, total leukocyte count, and differential leukocyte count were determined.

To determine PCV, 1 plain microhematocrit tube (Fisherbrand, Fisher Scientific, Pittsburgh, PA, USA) was filled from each anticoagulated sample and then centrifuged for 4 minutes at 1452*g*. The PCV to the nearest percentage was read by using a microhematocrit chart. The tubes were then broken at the buffy coat, and the plasma protein concentration was determined by refractometer (Leica Microsystems Inc, Buffalo, NY, USA).[14] The total leukocyte concentration was calculated by an indirect method, the eosinophil Unopette method (Unopette, Becton Dickinson) and by hemacytometer (Improved Neubauer Hemacytometer, Bright-Line, Cambridge Instruments Inc, Buffalo, NY, USA). The differential count was calculated from a Wright stained blood smear.[15–17] The blood smear was prepared by mixing 5 drops of the blood sample and 1 drop of 22% bovine albumin (Immucor Inc, Norcross, GA, USA). Total white blood cell (WBC) counts were calculated by the

formula: (total WBC per 9 squares of both sides of the hemacytometer) × 1.1 × 16 × 100/(percentage of heterophils + eosinophils). Absolute leukocyte counts were then calculated by multiplying this number with the percentages obtained from the modified Wright stained blood smears. The blood smears were evaluated for cell preservation by determining the presence of smudged cells or free nuclei. The effect of the anticoagulant on the cell-staining characteristics was subjectively determined by evaluating the eosinophilic, basophilic, and metachromatic staining patterns of the leukocyte granules and the intensity of nuclear chromatin staining in the erythrocytes, leukocytes, or thrombocytes. The presence of thrombocyte clumping and cell morphology and staining characteristics were evaluated microscopically at ×1000.

The distribution of the hematologic data was evaluated for normality by the Shapiro-Wilk test. The mean, SD, and minimum and maximum values were reported for normally distributed data, whereas the median, deciles D1 and D9, and minimum and maximum values were reported for non-normally distributed data. Nonparametric data were log transformed for parametric analysis (eosinophil and basophil counts). A paired sample *t* test was used to determine if differences occurred between the 2 anticoagulants for continuous variables, whereas the Fisher exact test was used to evaluate the same comparison between categorical data (thrombocyte clumping). Statistical significance was set at $P \leq .05$. A post hoc power was calculated if $P < .10$. Data were analyzed by using SPSS 11.0 (SPSS Inc, Chicago, IL, USA).

The level of agreement between samples was determined by the Bland-Altman method. For this analysis, bias was defined as the mean difference between results of the 2 methods, and the limits of agreement were calculated as mean bias ± 1.96 × SD. MedCalc (MedCalc Software, Mariakerke, Belgium) was used to analyze the data. For categorical data, the Cohen κ test was used to measure agreement.

Results

Results showed significant differences between the 2 anticoagulant samples in PCV measurements ($P < .001$), plasma protein concentration ($P < .001$), and lymphocyte counts ($P < .05$). Plasma protein and PCV values were significantly lower in lithium heparin samples, whereas lymphocyte counts were significantly higher in lithium heparin samples (Table 1). Heterophil counts between the 2 anticoagulants were not significantly different ($P = .08$). Although the $P < .10$, the observed power for the comparison between the methods used for heterophil counts was .69. No significant differences were detected for the other hematologic parameters. The level of agreement between the anticoagulants for heterophils (mean bias: −0.31; limits of agreement: −1.64 to 1.01), eosinophils (mean bias: 0.03; limits of agreement: −0.46 to 0.53), and basophils (mean bias: −0.02; limits of agreement: −0.60 to 0.56) were good; whereas, the level of agreement for PCV (mean bias: −3.5; limits of agreement: −7.06 to 0.07), WBC (mean bias: 0.98; limits of agreement: −4.10 to 6.07), lymphocytes (mean bias: 1.11; limits of agreement: −2.56 to 4.79), monocytes (mean bias: 0.18; limits of agreement: −1.15 to 1.52), and plasma protein (mean bias: −0.635; limits of agreement: −1.2 to −0.01) were considered only moderate.

There were small to large clusters of intact thrombocytes in the monolayer area of the blood smears in 65% (13/20) of the K_2EDTA samples and 95% (19/20) of the lithium heparin samples; however, this difference was not significant ($P = .35$). Five percent (1/20) of the birds had no thrombocyte clumping in either EDTA or heparin samples. Sixty percent (12/20) of the birds had thrombocyte clumps in both EDTA and heparin samples. The κ statistic for the comparison of thrombocyte clumping between the 2 anticoagulants was 0.18. Subjectively, there were no apparent differences in cell preservation and staining characteristics between the anticoagulants.

Discussion

In this study, the hematologic values in blood samples from Hispaniolan Amazon parrots collected in lithium heparin and in K_2EDTA were compared. The level of agreement between the samples was lowest for the plasma protein concentration, PCV, and lymphocyte count, and were highest for the remaining blood cell types. The limits of agreement in the results of the different values compared are subjectively small enough for us to be confident that lithium heparin can be used for routine complete blood counts in a clinical setting. The same anticoagulant should be used to follow trends within the same patient, especially when considering plasma protein, PCV, and lymphocyte count.

Regression or correlation coefficients have been used historically to measure the level of agreement

Table 1. Descriptive measures (mean, SD, and range; or median and deciles D1 and D9 [eosinophils, basophils]) for hematologic values by anticoagulant, lithium heparin, or dipotassium ethylenediaminetetraacetic (K_2EDTA) (N = 20).

Anticoagulant	Plasma protein (g/dl)	Packed cell volume (%)	White blood cell count ($\times 10^3/\mu l$)	Heterophils ($\times 10^3/\mu L$)	Lymphocytes ($\times 10^3/\mu l$)	Monocytes ($\times 10^3/\mu l$)	Eosinophils ($\times 10^3/\mu l$)[a]	Basophils ($\times 10^3/\mu l$)[a]
K_2-EDTA	5.7[b] ± 0.5	53.0[b] ± 3.8	10.4 ± 3.1	5.2 ± 1.8	3.6[b] ± 2.0	1.1 ± 0.5	0.1 ± 0.0–0.6	0.1 ± 0.0-0.5
	(4.9–6.5)	(47.0–60.0)	(5.8–15.9)	(2.3–7.4)	(1.4–8.1)	(0.3–2.3)	(0.0–1.0)	(0.0–1.0)
Lithium heparin	5.2[b] ± 0.3	50.3[b] ± 3.5	10.9 ± 2.9	5.0 ± 1.6	4.5[b] ± 1.6	1.0 ± 0.4	0.1 ± 0.0–0.7	0.2 ± 0.0–1.0
	(4.0–6.0)	(46.0–57.0)	(5.1–14.4)	(1.4–6.8)	(1.4–6.8)	(0.3–1.8)	(0.0–1.0)	(0.0–1.1)

[a] Median and deciles D1 and D9.
[b] $P < .05$.

for a continuous biologic parameter; however, neither of these methods is optimal.[18–21] The technique considered most appropriate for measuring the level of agreement is the Bland-Altman method and was the technique used in this study.[19,21] The level of agreement between the 2 methods is limited by the precision (ie, repeatability of the results) of the methods compared. If 1 method has poor precision, the agreement between the 2 methods will likely be poor.[19] The Unopette system is a standardized method, with a known coefficient of variation (6.8%), for determining the leukocyte concentration.[22] There are no other reports regarding precision for other hematologic values (eg, PCV, plasma protein) calculated in this study.

The values for PCV were significantly lower when using lithium heparin than with K_2EDTA. These differences could not be attributed to hemolysis, because hemolysis was not appreciated in any of the anticoagulated blood samples of our study. In studies of human blood samples, K_2EDTA outside of the optimum concentration (1–2 mg/ml blood) causes a progressive decrease in the estimate of PCV values.[23,24] The effect of K_2-EDTA is attributed to the reduction of RBC volume caused by the anticoagulant cation in the plasmatic osmotic pressure.[23] Heparin alone, however, causes little alteration in corpuscular size and is considered a more suitable anticoagulant, because varying the concentration has little effect on the PCV values.[23,24] In our study, the tubes were filled to an appropriate volume according to manufacturer instructions; however, the use of lithium heparin, instead of heparin, could account for the difference in results.

Plasma protein values were significantly lower in samples anticoagulated with lithium heparin than in those with K_2EDTA. The use of EDTA can affect the refractometric reading of proteins,[25,26] whereas similar effects have not been reported with heparin. In this study, total protein values were estimated by refractometry, approximating the total solids measured. The accuracy of the refractometric method to determine total protein concentration in avian blood is considered poor.[27] Because of the higher glucose and lower total protein concentrations in birds, correlation of results from the refractometer and the biuret methods may not be possible in some species.[28] Refractometry should be considered a rapid method for determining an estimate of the fluid protein. Ideally, total protein concentrations should be determined by plasma protein electrophoresis.

In this study, no significant differences were observed in the total leukocyte count between anticoagulants. However, the use of heparinized blood samples has been considered to result in erroneous counts because of leukocyte and thrombocyte clumping and improper staining of cells.[29] In 1 study in macaws (*Ara* species), no significant differences were found in results from lithium heparin samples at 3 and 24 hours after sampling, whereas, at 12 hours, WBC and RBC lysis were markedly increased in K_3EDTA-anticoagulated samples.[1] Because heparin-coated syringes were used in that study, however, the effect of the individual coagulants could not be fully ascertained. In several reptile species, significantly higher values for total WBC counts and most differential leukocyte counts were found in EDTA samples,[11,12] whereas, in another study, there were no differences in results with different anticoagulants.[1]

The leukocyte counts were determined by an indirect method with phloxine B solution. Phloxine B stains only the acidophilic granulocytes (heterophils and eosinophils). Basophils can also be identified by using increased contrast when counting the leukocytes. The count was corrected for mononuclear cells once the differential was made. The slight decrease in the granulocyte numbers in lithium heparin samples, which would result in an overestimate of the mononuclear cells, could explain the higher lymphocyte count in those samples. Otherwise, no significant differences were found in heterophil, monocyte, or basophil numbers in the samples stored in K_2EDTA or heparin.

Heparin is reported to alter the nuclear staining of blood cells and to cause changes in avian blood cell morphology.[30] This widely quoted study stated that heparin has a negative effect on morphology and staining was based on blood samples from domestic chickens that were citrated, washed, and subsequently exposed to high concentrations of heparin and that were read within 8 hours.[31] In our study, these changes were not evident on blood-smear examinations from heparin samples when compared with those from EDTA samples. Also, samples in our study were processed within 2 hours. Further studies are needed to characterize the temporal effects of individual anticoagulants. Thrombocyte clumping was increased in the lithium heparin samples, and the agreement was poor with K_2EDTA samples, indicating that the thrombocyte count may be affected by the choice of anticoagulant. However, a thrombocyte count was not done in this study.

Although K_3EDTA was used in a study conducted in macaws,[1] K_2EDTA was used in our study. The differences in these 2 formulation were studied in human medicine, with the conclusion that the differences observed by using K_3EDTA glass tubes versus K_2EDTA plastic tubes were unlikely to be of any clinical significance.[32]

Subjectively, no negative effects on morphology and staining of blood cells were apparent in smears from heparin samples compared with K_2EDTA samples. The poor level of agreement between anticoagulants with the increase in thrombocyte clumping in lithium heparin samples indicates that the use of lithium heparin as an anticoagulant may affect thrombocyte count. The limits of agreement within the remaining values that were compared are small enough to be confident that lithium heparin can be used for routine CBCs in Hispaniolan Amazon parrots when the samples are processed within 2 hours. To follow trends within the same patient, the same anticoagulant should be used for serial comparisons, especially when considering plasma protein concentration, PCV, and lymphocyte count. Further studies are needed to evaluate the temporal effect of anticoagulants on the hematologic values in avian species.

References

1. Harr KE, Raskin RE, Heard DJ. Temporal effects of 3 commonly used anticoagulants on hematologic and biochemical variables in blood samples from macaws and Burmese pythons. *Vet Clin Path*. 2005;34:383–388.
2. Knoll JS. Clinical automated hematology systems. In: Feldman BF, Zinkl JG, Jain NC, eds. *Schalm's Veterinary Hematology,* 5th ed. Philadelphia, PA: Lippincott, Williams, & Wilkins; 2000:3–11.
3. Jain NC. Hematologic techniques. In: Jain NC, ed. *Veterinary Hematology*. Philadelphia, PA: Lea and Febiger; 1986:23–86.
4. Samour J. Diagnostic value of hematology. In: Harrison GJ, Lightfoot TL, eds. *Clinical Avian Medicine*. Palm Beach, FL: Spix Publishing; 2006:587–610.
5. Campbell TW. *Avian Hematology and Cytology.* 2nd ed. Ames, Iowa: Iowa State University Press; 1995.
6. Lumeij J. Avian clinical pathology. *Vet Q*. 1987;9:249–254.
7. Fudge AM. Avian complete blood count. In: Fudge AM, ed. *Laboratory Medicine, Avian and Exotic Pets*. Philadelphia, PA: WB Saunders; 2000:9–18.

8. Hattingh J, Smith EM. Anticoagulants for avian and reptilian blood: heparin and EDTA. *Pflugers Arch*. 1976;363:267–269.
9. Fourie FlR. Effects of anticoagulants on the hematocrit, osmolarity and pH of avian blood. *Poulty Sci*. 1977;56:1842–1846.
10. Uko OJ, Ataja AM. Effects of anticoagulants and storage (4 degrees C) on packed cell volume (PCV) of Nigerian domestic fowl (*Gallus domesticus*) and guinea fowl (*Numida meleagris*). *Br Poult Sci*. 1996;37:997–1002.
11. Hanley CS, Hernandez-Divers SJ, Bush S, Latimer KS. Comparison of the effect of dipotassium ethylenediaminetetraacetic acid and lithium heparin on hematologic values in the green iguana (*Iguana iguana*). *J Zoo Wildl Med*. 2004;35: 328–332.
12. Martinez-Jimenez D, Hernandez-Divers SJ, Floyd TM, et al. Comparison of the effects of dipotassium ethylenediaminetetraacetic acid and lithium heparin on hematologic values in yellow-blotched map turtles (*Graptemys flavimaculata*). *J Herp Med Surg*. 2007;17:36–41.
13. Muro J, Cuenca R, Pastor J, et al. Effects of lithium heparin and tripotassium EDTA on hematologic values of Hermann's tortoises (*Testudo hermanni*). *J Zoo Wildl Med*. 1998;29:40–44.
14. Hochleithner M. Biochemistries. In: Ritchie BW, Harrison GJ, Harrison LR, eds. *Avian Medicine: Principles and Application*. Lake Worth, FL: Wingers Publishing; 1994:223–245.
15. Wiseman BK. An improved method for obtaining the total white blood cell count in avian blood. *Proc Soc Exp Biol Med*. 1931;28:1030–1033.
16. Costello RT. A Unopette for eosinophil counts. *Am J Clin Pathol*. 1970;54:249–250.
17. Ferris M, Bacha WJ. A new method for the identification and enumeration of chicken heterophils and eosinophils. *Avian Dis*. 1984;28:179–182.
18. Seed P. Comparing several methods of measuring the same quality. *Stata Technical Bulletin*. 2000;10: 2–9.
19. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet*. 1986;1:307–310.
20. Glantz S. How to test for trends. In: *Primer of Biostatistics*. New York: McGraw-Hill; 2005: 225–230.
21. Bland JM, Altman DG. Measuring agreement in method comparison studies. *Stat Method Med Res*. 1999;8:135–160.
22. Dein JF, Wilson A, Fischer D, Langenberg P. Avian leukocyte counting using the hemocytometer. *J Zoo Wild Med*. 1994;25:432–437.
23. Dubin S, Piszczek J, Beard R, Schmukler R. Effects of anticoagulants on packed cell volume measurement. *Lab Anim Sci*. 1976;26:586–591.
24. Pennock C, Jones K. Effect of ethylene-diamine-tetra-acetic (dipotassium salt) and heparin on the estimation of packed cell volume. *J Clin Pathol*. 1966;19:196–199.
25. George JW. The usefulness and limitations of hand-held refractometers in veterinary laboratory medicine: an historical and technical review. *Vet Clin Pathol*. 2001;30:201–210.
26. Dubin S, Hunt P. Effect of anticoagulants and glucose on refractometric estimation of protein in canine and rabbit plasma. *Lab Anim Sci*. 1978;28:541–544.
27. Lumeij JT, de Bruijne JJ. Evaluation of refractometric method for the determination of total protein in avian plasma or serum. *Avian Pathol*. 1985;14:441–444.
28. Lumeij JT, Maclean B. Total protein determination in pigeon plasma and serum: comparison of refractometric methods with the biuret method. *J Avian Med Surg*. 1996;10:150–152.
29. Campbell TW, Ellis CK. Hematology of birds. *Avian and Exotic Hematology and Cytology*. 3rd ed. Ames, Iowa: Iowa State University Press; 2007:3–50.
30. Freidlin PJ. Destructive effects of heparin on avian erythrocytes. *Avian Pathol*. 1985;14:531–536.
31. Walberg J. White blood cell counting techniques in birds. *Semin Avian Exotic Pet Med*. 2001;10:72–76.
32. Van Cott EM, Lewandrowski KB, Patel S, et al. Comparison of glass K_3EDTA versus plastic K_2EDTA blood-drawing tubes for complete blood counts, reticulocyte counts, and white blood cell differentials. *Lab Hematol*. 2003;9:10–14.