Development of a technique for quantification of reticulocytes and assessment of erythrocyte regenerative capacity in birds

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Objective—To develop a reticulocyte classification scheme, optimize an avian reticulocyte staining protocol, and compare the percentages of reticulocyte types with polychromatophil percentage in blood samples from birds.

Sample Population—Blood samples from a red-tailed hawk and 31 ill birds.

Procedures—A single blood sample obtained from a red-tailed hawk (Buteo jamaicensis) was used to optimize the staining protocol. For optimization of the staining protocol, 4 dilutions of whole blood with new methylene blue stain and 4 incubation times were evaluated. From samples submitted for avian CBCs, EDTA-anticoagulated whole blood samples from 31 ill birds were randomly selected and examined to compare polychromatophil and reticulocyte percentages. Reticulocyte staining was performed in all samples by use of a 1:3 (whole blood to new methylene blue) dilution with incubation for 10 minutes at room temperature (approx 22°C); reticulocytes were assessed as a percentage of 1,000 RBCs by 2 independent observers. In Wright-Giemsa–stained blood smears, a polychromatophil percentage was similarly determined.

Results—4 avian reticulocyte types were defined: ring-form reticulocytes, aggregate reticulocytes, and 2 subcategories of punctate reticulocytes. A reticulocyte-staining protocol was optimized. Interobserver and intraobserver variations in assessment of reticulocyte and polychromatophil percentages were not significant. A strong positive correlation (Spearman coefficient of rank correlation \( \rho = 0.978 \)) was identified between the percentage of polychromatophils and the percentage of ring-form reticulocytes.


Anemia is a common clinicopathologic abnormality detected in avian species. It has been estimated that the frequency of anemia in birds, based on samples submitted for CBCs, is 12.7%. The initial diagnostic approach to anemia in both mammalian and avian patients is to first classify the anemia as regenerative or nonregenerative on the basis of the quantity of reticulocytes. For several mammalian species, reticulocyte counts are quantitatively assessed by use of automated analyzers. In contrast, avian erythrocyte regenerative responses are evaluated via semiquantitative assessment of polychromasia or estimation of reticulocyte percentages because the presence of nuclei in avian erythrocytes precludes the use of automated analyzers for quantitative reticulocyte counting.

Consequently, a problem associated with assessment of erythroid regeneration in birds is determining the best method for manually counting avian reticulocytes. There is general agreement that supravital stains such as NMB can be used to identify avian reticulocytes. However, investigators have reported detection of basophilic reticulum in a high percentage of avian erythrocytes following supravital staining; counting all cells containing any visible reticulum has yielded high reticulocyte percentages (some > 90%) in both healthy birds and birds with anemia induced via nutritional alterations. Clearly, more specific morphologic criteria to define reticulocytes are needed. Two commonly used classification schemes recommend counting either all cells with > 4 or > 5 distinct aggregates of reticulum; another scheme recommends counting all cells with a complete ring of reticulum that encircles the nucleus. This latter scheme also has been recommended in several current clinical textbooks, but as yet, a consensus on avian reticulocyte classification has not been reached.

**Abbreviations**

<table>
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<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>CV</td>
<td>Coefficient of variation</td>
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<td>IEC</td>
<td>Immature erythroid cell</td>
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<td>NMB</td>
<td>New methylene blue</td>
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Received May 26, 2007.
Accepted November 7, 2007.
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In recent years, a variety of methods for counting avian reticulocytes has been used, and often the reports fail to define the method of reticulocyte counting. Additionally, the terms reticulocyte and polychromatophil are sometimes used interchangeably. This lack of uniformity in avian reticulocyte assessment prevents comparison of results among studies and has been identified as a likely cause of apparent variation in reticulocyte counts in chickens. A similar lack of uniformity in avian reticulocyte assessment exists among veterinary diagnostic laboratories. Many laboratories report only a semiquantitative estimate of polychromasia, whereas others report reticulocyte percentages (derived by use of various methods); few laboratories have established reference intervals for either variable.

The purpose of the study reported here was to develop a reticulocyte classification scheme, optimize an avian reticulocyte staining protocol, and compare the percentages of reticulocyte types with polychromatophil percentage in blood samples from birds. Additionally, determination of a reference interval for polychromatophils in psittacines was undertaken.

**Materials and Methods**

**Definition of reticulocyte types**—Four types of reticulocytes were defined on the basis of the reticulum pattern revealed via supravital NMB staining (Figure 1). The reticulocyte classification categories were as follows: punctate-1 (<10 dots of stained reticulum); punctate-2 (≥10 dots of stained reticulum); aggregate (any aggregates of stained reticulum, distributed diffusely within the cytoplasm); and ring-form (aggregates of stained reticulum that formed a ring—contiguous or discontinuous—around at least half of the reticulocyte nucleus).

**Comparison of reticulocyte-staining methods and evaluation of sample storage**—A single 4-mL sample of whole blood was collected (immediately prior to euthanasia) from an injured red-tailed hawk that was brought to the veterinary medical hospital at the University of California, Davis; EDTA was used as the anticoagulant for the sample. The whole blood sample was diluted with NMB stain (1:1, 1:2, 1:3, and 1:4 [blood to stain]). Whole blood volumes of 10 to 20 µL were placed into plastic bullet tubes, and appropriate volumes of stain were added. The tubes were capped, mixed by repeated inversion, and allowed to incubate at room temperature (approx 22°C) for 5, 10, 30, or 60 minutes. Repeated inversion of the tubes was again performed before a direct smear of the contents of each was made. Sixteen smears (1 from each of the 4 diluted samples that underwent each incubation period) were evaluated by 1 observer (MPS). By use of the aforementioned reticulocyte classification scheme, the percentage of each reticulocyte type among 1,000 RBCs examined on each smear was determined.

A dilution of 1:3 (blood to stain) and a 10-minute incubation time were used to evaluate reticulocyte staining after 4, 12, 24, and 48 hours of storage of the EDTA whole blood sample at 4°C. Staining quality was subjectively evaluated for all smears, and the percentage of each reticulocyte type among 1,000 RBCs examined on each smear was determined.

Degenerated erythrocytes, defined as cells with fragmented nuclear chromatin or deeply basophilic cytoplasm (or both), were counted separately when observed.

**Comparison of polychromatophil and reticulocyte percentages**—Samples of blood (anticoagulated with EDTA) from 31 birds that were examined at the University of California, Davis; EDTA was used as the anticoagulant for the sample. The whole blood sample was diluted with NMB stain (1:1, 1:2, 1:3, and 1:4 [blood to stain]) from 31 birds that were examined at the University of California, Davis; EDTA was used as the anticoagulant for the sample. The whole blood sample was diluted with NMB stain (1:1, 1:2, 1:3, and 1:4 [blood to stain]). Whole blood volumes of 10 to 20 µL were placed into plastic bullet tubes, and appropriate volumes of stain were added. The tubes were capped, mixed by repeated inversion, and allowed to incubate at room temperature (approx 22°C) for 5, 10, 30, or 60 minutes. Repeated inversion of the tubes was again performed before a direct smear of the contents of each was made. Sixteen smears (1 from each of the 4 diluted samples that underwent each incubation period) were evaluated by 1 observer (MPS). By use of the aforementioned reticulocyte classification scheme, the percentage of each reticulocyte type among 1,000 RBCs examined on each smear was determined.

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vetinary medical teaching hospital were randomly selected from all avian blood samples submitted to the clinical pathology laboratory for routine CBCs. This group of birds included Psittaciformes (n = 16), Anseriformes (3), Falconiformes (7), and Strigiformes (3). Wright-Giemsa–stained smears were prepared from each blood sample as part of the CBC. An aliquot of whole blood (10 to 20 µL) was taken from each sample and added to NMB stain in a 1:3 dilution; samples were incubated at room temperature for 10 minutes, and a direct smear was prepared. Two observers (JLJ and MPS [observers 1 and 2]) each performed differential counts on 1,000 cells on the Wright-Giemsa– and the NMB-stained smears. Normochromic erythrocytes, polychromatophilic erythrocytes, and IECs (defined as round erythroid cells with basophilic cytoplasm) were counted in the Wright-Giemsa–stained smears (Figure 2). For comparison of different combinations of cells to reticulocytes, an adjusted polychromatophil percentage was calculated (ie, IECs were excluded from polychromatophil and total RBC counts) to express polychromatophils as a percentage of the combined number of polychromatophils and mature RBCs. A combined percentage of polychromatophils and IECs, which expressed all immature erythrocytes as a percentage of all erythroid cells, was also calculated. The 4 reticulocyte types (punctate-1, punctate-2, aggregate, and ring-form) were each counted in the NMB–stained smears and expressed as percentages. A combined percentage of aggregate and ring-form reticulocytes was also calculated.

Comparisons were made between adjusted polychromatophil percentage and percentages of each of the 4 reticulocyte types and also the combined percentage of aggregate and ring-form reticulocytes. Comparisons were also made between the combined percentage of polychromatophils and IECs and percentages of the 4 reticulocyte types and also the combined percentage of aggregate and ring-form reticulocytes. To evaluate the potential for observer bias, the differential counts on 1,000 cells were repeated on all NMB-stained smears by both observers at the conclusion of the study.

Generation of a reference interval for polychromatophils in healthy psittacines—Clinic records from January 2003 to January 2007 were searched electronically for psittacines that underwent a routine wellness examination. A bird was considered healthy if no clinical abnormalities or other evidence of disease was detected via physical examination and if results of a CBC and plasma biochemical analyses were within published reference intervals for healthy psittacines.1 Birds were excluded if they did not meet all of those criteria or if they were < 6 months old. Differential counts on 1,000 RBCs were performed on archived Wright-Giemsa–stained smears of blood from the birds that met the criteria for inclusion. Percentages of polychromatophils and IECs were recorded for each smear. Absolute polychromatophil counts were calculated by multiplying the polychromatophil percentage by the total RBC count (manual leukocyte counts were subtracted from the total nucleated cell count obtained from the hematology analyzer7 to obtain the total RBC count).

**Statistical analysis**—Descriptive statistics were generated for data from the comparison of staining methods.4 Interobserver and intraobserver variation in the percentage of ring-form reticulocytes was assessed via repeated-measures ANOVA.5 Polychromatophil and reticulocyte percentages were compared by use of a Spearman rank correlation procedure.6 Deming regression was used to compare polychromatophil and ring-form reticulocyte percentages recorded by both observers.6 The distributions of both the percentage and absolute polychromatophil data were evaluated by use of the D’Agostino-Pearson test.6 A value of P < 0.05 was considered significant.

**Results**

Comparison of reticulocyte-staining methods and evaluation of storage—Staining quality of the 4 smears made from blood samples that were diluted 1:1 with NMB stain and incubated at room temperature for 5, 10, 30, or 60 minutes was unacceptable for differential counting because the reticulum was often faintly stained, thereby precluding differentiation of various reticulocyte types. Staining quality of the remaining 12 smears was assessed as acceptable for differential

<table>
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<th>Variable</th>
<th>Punctate-1† (%)</th>
<th>Punctate-2† (%)</th>
<th>Aggregate† (%)</th>
<th>Ring-form† (%)</th>
<th>Aggregate and ring-form (%)</th>
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<tr>
<td>Polychromatophil (adjusted %)</td>
<td>0.490 (0.003)</td>
<td>0.192 (0.02)</td>
<td>0.377 (0.04)</td>
<td>0.978 (0.001)</td>
<td>0.651 (&lt; 0.001)</td>
</tr>
<tr>
<td>Polychromatophil and IEC (%)</td>
<td>0.498 (0.016)</td>
<td>0.180 (0.02)</td>
<td>0.370 (0.002)</td>
<td>0.976 (0.001)</td>
<td>0.647 (&lt; 0.001)</td>
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Direct smear were prepared from samples that were stained with NMB stain in a 1:3 dilution (blood to stain) and incubated at room temperature (approx 22°C) for 10 minutes. Wright-Giemsa–stained smears were also available from routine CBCs performed for each bird. Two observers each performed differential counts on 1,000 cells on the Wright-Giemsa– and the NMB-stained smears. *Calculated by exclusion of IECs from counts. †Four types of reticulocytes were defined on the basis of the reticulum pattern revealed via supravital NMB staining as follows: punctate-1 (< 10 dots of stained reticulum); punctate-2 (> 10 dots of stained reticulum); aggregate (any aggregates of stained reticulum, distributed diffusely within the cytoplasm); and ring-form (aggregates of stained reticulum that formed a ring—contiguous or discontinuous—around at least half of the reticulocyte nucleus). A value of P < 0.05 was considered significant for the correlation coefficient.
Comparison of polychromatophil and reticulocyte percentages—On the basis of data collected from examination of Wright-Giemsa– and NMB-stained blood smears from 31 birds by the 2 observers, inter- and intraobserver variations were first calculated for ring-form reticulocyte types. Calculated F (test statistic for ratio of mean square numbers) values were low, and P values were >0.05 for all comparisons (for interobserver variation, F = 0.072 and P = 0.790; for intraindividual variation for observer 1, F = 0.404 and P = 0.530; and for intraindividual variation for observer 2, F = 0.083 and P = 0.775). Because these results indicated a lack of significant interobserver or intraindividual variation, differential percentages for the 2 observers were calculated and used for further comparisons.

Ten comparisons were assessed by use of Spearman rank correlation coefficient (Table 1). Correlation between adjusted polychromatophil percentage and the percentage of ring-form reticulocytes was strongest (ρ = 0.978; P < 0.001). Correlation between the combined percentage of polychromatophils and IECs and the percentage of ring-form reticulocytes was only slightly less strong (ρ = 0.976; P < 0.001). These 2 comparisons were plotted, and regression line equations were obtained (Figure 3). Correlation coefficients were substantially lower for the other 8 comparisons. The Deming regression model was used to compare data generated separately by each observer for polychromatophil percentage with data generated for ring-form reticulocyte percentage, and CVs were calculated for both measurement techniques. The CV for polychromatophil quantitation was 19.44%, whereas the CV for ring-form reticulocyte quantitation was 8.43%. The 95% confidence intervals were −0.017 to 0.003 for regression line intercept and 0.910 to 1.160 for regression line slope.

Generation of a reference interval for polychromatophils in healthy psittacines—On review of clinic records, 44 birds were defined as healthy adult psittacines according to the defined criteria. Differential counts on 1,000 RBCs were performed on archived smears of blood from these birds. Data for both the percentage and absolute number of polychromatophils were normally distributed, and no outliers were identified; thus, the reference interval for each was defined as the mean ± 2 SD value. The reference interval for percentage polychromatophils was 0.60% to 8.00% (mean, 4.36%; CV, 3.20%; punctate-2 reticulocytes, 26.11 ± 3.20%; punctate-2 reticulocytes, 19.97 ± 4.36%; aggregate reticulocytes, 47.73 ± 4.40%; and ring-form reticulocytes, 6.19 ± 0.33%. The CV values for the 4 reticulocyte types were as follows: punctate-1 reticulocytes, 12.25%; punctate-2 reticulocytes, 21.82%; aggregate reticulocytes, 9.22%; and ring-form reticulocytes, 5.61%. The CVs were subjectively accepted as acceptable for reticulocyte percentages for blood samples stored at 4°C for 4, 12, and 24 hours prior to staining, with CV values of 9.10%, 13.44%, 6.87%, and 18.16% for ring-form, aggregate, punctate-2, and punctate-1 reticulocyte types, respectively. Degenerated erythrocytes comprised 0.6% of erythrocytes in the sample stored for 24 hours. Many of the erythrocytes in the sample stored for 48 hours prior to staining had glassy, deeply basophilic cytoplasm, which precluded reticulocyte evaluation; the sample quality was deemed unacceptable for reticulocyte counting.

In the present study, a method of avian reticulocyte counting that was accurately reproduced by different observers was investigated. The 4 categories of reticulocyte morphologic types were representative of classification schemes used by previous investigators. In our study, these criteria were more clearly defined, and in routinely stained blood smears, reticulocyte classifications were correlated with percentage of polychromatophils. For the 2 observers involved in the study, intraindividual CV for ring-form reticulocytes was low (5.61%), which suggested that counting this specific type of reticulocyte may be associated with a greater degree of reproducibility than that associated with counting any of the other 3 reticulocyte types. The results of our comparisons further indicated that the ring-form reticulocyte percentage correlated strongly...
with the polychromatophil percentage. Counting of ring-form reticulocytes is therefore recommended as the best method for avian reticulocyte quantification, given both the strong correlation between ring-form reticulocytes and polychromatophils and the higher degree of precision in counting ring forms versus other types of reticulocytes.

A separate category of IECs was created to include erythroid cells with a morphologic appearance that was more immature than that of polychromatophils; these cells are considered analogous to nucleated RBCs in mammalian blood. On the basis of findings in supravital stained avian bone marrow,7 the staining pattern of IECs should be similar to that of ring-form reticulocytes, and we considered the potential impact of inclusion or exclusion of these cells in our study via comparisons with an adjusted polychromatophil percentage (with exclusion of IECs) and with the combined percentage of polychromatophils and IECs. Both polychromatophil categories quantitatively correlated strongly with ring-form reticulocytes, with only a slight decrease in correlation strength when IECs were included. Immature erythroid cells comprised 0.0% to 5.0% of all RBCs in the samples included in our study, which was a wide range of values. The practical implication of this finding is that inclusion or exclusion of IECs in a polychromatophil reference interval appears to have a minimal, and likely clinically unimportant, effect on the percentage of ring-form reticulocytes.

In addition to percentages of each reticulocyte type, a combined percentage of aggregate and ring-form reticulocytes was also compared with the percentage of polychromatophils. This fifth category was added because ring-form reticulocytes arguably represent a subgroup of aggregate reticulocytes, such that a category of what might be considered all aggregate reticulocytes could show the strongest correlation with polychromatophils in avian species, as is true in domestic cats.11 However, strong correlation between the percentage of polychromatophils and the combined percentage of aggregate and ring-form reticulocytes was not detected in our study.

Lack of uniformity in counting and reporting of avian reticulocytes has been identified as a likely cause of variation in published reticulocyte counts in chickens.10 The data from the present study supported this theory. In the 1950s and 1960s, reported values for reticulocytes in clinically normal humans also varied markedly, and several authors attributed this to a lack of uniform criteria for identification of reticulocytes.13 Standardization of a uniform reticulocyte-counting technique should reduce variation in proposed reference intervals in avian species, as has been the case for mammalian species.

Supravital staining of avian bone marrow has revealed the presence of a perinuclear ring of reticulum in cells in the early stages of erythroid cell development.7 Avian erythrocytes do not extrude their nucleus during maturation as do mammalian erythrocytes, but a maturation stage similar to that of a late metarubocyte was believed to be indicated by dissolution of the perinuclear ring of reticulum into a diffuse distribution of granular reticulum throughout the cytoplasm.7 The results of our study suggest that this transformation from a perinuclear to a diffuse distribution of reticulum is more likely to be analogous to the maturation of a polychromatophil to a mature erythrocyte because the percentage of ring-form reticulocytes (ie, those with perinuclear granulation) quantitatively correlated much more strongly with the percentage of polychromatophils than did the percentage of aggregate reticulocytes (ie, those with diffusely distributed granulation) or the combined percentage of aggregate and ring-form reticulocytes.

In the present study, the optimal avian reticulocyte staining protocol involved a 1:3 dilution of whole blood to NMB stain with an incubation time of 10 minutes. Ratios of whole blood to supravital stain ranging from 1:1 to 1:3 have been used previously for staining of blood from both avian and mammalian species.6,14 Both the type and concentration of the supravital stain used must be taken into account when comparing staining methods. The 1:3 dilution was selected as optimal in our study because cytoplasmic staining was better defined at this dilution than at lower dilutions.

Refrigerated storage of whole blood for as long as 24 hours minimally affected reticulocyte staining and counting in our study, although there was a low percentage (0.6%) of erythrocytes that were degenerated in appearance and could not be included in the differential count. After 48 hours of refrigerated storage, many cells were degenerated and deeply stained, and reticulocyte counting could not be performed. Therefore, our recommendation is to store avian whole blood in a refrigerator for no more than 24 hours prior to reticulocyte staining and counting.

Hematology reference intervals for healthy birds can be difficult to establish, in part because of population sample size and sample volume limitations, and in part because of the wide phylogenetic diversity of avian patients. Because of the strong correlation between percentages of polychromatophils and ring-form reticulocytes detected in the present study, which included blood samples from birds in 4 different orders, we proposed that a polychromatophil reference interval could be used as a substitute for a reticulocyte (ring-form) reference interval. The ability to assess erythrocyte regeneration from examination of routinely stained blood smears would save time and avoid the need for collection of an additional sample volume for a reticulocyte count. The polychromatophil percentage reference interval generated in our study (0.60% to 8.00%) was consistent with published estimates13 of polychromasia in healthy birds. An absolute polychromatophil reference interval was also generated, but no published estimates of this interval could be found for comparison. Because of the proportionate bias between percentages of polychromatophils and ring-form reticulocytes, the polychromatophil reference interval should be used only as an estimate of, rather than as a replacement for, a reference interval for ring-form reticulocytes. In addition, interobserver CV in the present study was considerably higher for counts of polychromatophils than it was for counts of ring-form reticulocytes (19.44% and 8.43%, respectively), which indicated that there was a greater degree of random error, and therefore greater imprecision, associated with counting polychromatophils than
counting ring-form reticulocytes. Observer detection of polychromatophilia is based on color alone and is therefore likely to be a more difficult and subjective assessment than detection of reticulocytes. Decreased interobserver agreement in blood smear evaluation has been correlated with color vision deficiencies, and a pattern-based system rather than a color-based system is clearly advantageous to a practitioner with color vision impairment. For these reasons, reticulocyte counting is the preferred laboratory method for evaluation of regenerative erythrocyte responses in birds, and reference intervals for ring-form reticulocytes should be developed by laboratories in which avian reticulocyte counts are performed.

a. Euthasol, Virbac Inc, Fort Worth, Tex.
b. 0.5% new methylene blue stain, Ricca Chemical Co, Arlington, Tex.
c. 7100 Aerospray stainer, Wescor Inc, Logan, Utah.
d. ADVIA 120, Bayer Diagnostics Division, Bayer Corp, Tarrytown, NY.
e. MedCalc for Windows, version 8.1.1, MedCalc Software, Mariakerke, Belgium.
f. SPSS for Windows, version 11.5, SPSS Inc, Chicago, Ill.

References