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Original Study

Comparison of 3 Leukogram Determination Methods in Avian Species: Phloxine B Stain, Blood Smear, and an Automated Analyzer

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Abstract: Because avian blood cells are nucleated, most automated methods used in mammalian species for total white blood cell (WBC) counts and differentials are considered inaccurate. Therefore, manual methods are routinely used in birds, although this could result in variations in methods across laboratories. The objective of this study was to evaluate and compare 3 methods of avian leukogram determination: a commercial phloxine B stain method (PB), estimation from a blood smear (EBS), and an automated analyzer (Cell Dyn 3500, [CD]). Leukograms from 23 avian blood samples were compared using these methods. All samples were evaluated once by 4 observers to assess the repeatability and precision of the manual methods (PB and EBS). Analyses with the CD method were repeated 5 times on 3 samples to evaluate repeatability. The WBC counts and differentials obtained with CD were compared to the 2 other methods by calculating intraclass correlation coefficients (ICC). Agreement between WBC counts from EBS and PB and between CD and PB was assessed with Bland-Altman plots. Results based on the CD analyzer correlated poorly with the other methods. When compared with the EBS method, ICCs ranged from 0–4.3% for heterophils, 0–12% for lymphocytes, 0–23.4% for monocytes, and were equal to 0% for eosinophils. When comparing the CD with PB, ICCs for WBC counts ranged from 85.9–91.5% among observers. High interobserver agreement was seen for the leukograms obtained with EBS (ICC = 92.9%). A good agreement was noted between EBS and PB for WBC counts (ICC = 69.5–81.3%). Bland Altman plots indicated good agreement for WBC counts between EBS and PB (slope P value = 0.52) and CD and PB (slope P value = 0.13). Although less precise than PB, EBS proved to be clinically useful and was both time and cost-efficient. The CD method does not seem adapted for avian leukocyte differentials.

Key words: avian, blood smear, hematology, method validation, phloxine B, automated analyzer

INTRODUCTION

Hematology is routinely used for health assessments, quarantine protocols, and to evaluate disease progression or response to treatment in avian patients. The complete blood cell count has been reported as a sensitive test to assess the health of an avian patient.[1] Currently, there is no reference standard for quantifying white blood cells (WBC) in avian species.[2] Most automated analyzers selectively differentiate leukocytes from erythrocytes by the absence or presence of a nucleus.[3] Typically, these automatic techniques initially involve the lysis of erythrocytes, so that the remaining leukocytes may then be identified based on size and granularity. Nucleated avian erythrocytes are more robust and resist lysis techniques, so harsher conditions are required to disrupt them and may damage leukocytes.[4] Additionally, free nuclei frequently remain in suspension after erythrocyte lysis, which will cause artifactual elevation of the resulting optical density and thus erroneously elevate the hemoglobin result. The presence of nucleated erythrocytes and thrombocytes, therefore, constitutes a major issue for leukogram determination in avian species.[2] The similar size of thrombocytes and lymphocytes, as well as the variability in size, shape, and dimension of blood cells across avian taxa, are additional sources of error with automated blood analyzers.[5] In poultry, automated flow cytometry analyzers have been used for total WBC counting. These techniques are primarily used to

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differentiate between heterophils and lymphocytes, with variable accuracy reported for differential white blood cell determination.[4,6,7] As automated techniques have not been extensively studied in other avian species, manual hematological methods are typically used, which results in increased inter-laboratory variability and a lack of standardization.[2]

Phloxine B (PB) staining is considered by many to be the most reliable method for leukocyte counts in avian species.[5,8–10] The PB diagnostic test is a semi-direct method in which only the granules of heterophils and eosinophils are stained. The WBC is then determined mathematically from a manual differential leukocyte estimate. Alternatively, the Natt-Herrick technique is considered a direct method in which all leukocytes are stained, and differential leukocyte counts are established manually using a hemocytometer. The PB is often preferred over the Natt-Herrick technique because multiple studies have reported greater precision with this method, likely due to the difficulty in differentiating lymphocytes from thrombocytes with the Natt-Herrick technique.[9,11] Despite this, estimation of WBC numbers on a blood smear (EBS) is frequently favored by clinicians because it requires only a small volume of blood and can be performed faster with routine laboratory equipment.[12] The Cell Dyn 3500 (CD; Abbott Laboratories, Abbott Park, IL, USA) is an automated analyzer that uses impedance and flow cytometry to identify leukocytes using optical measurements from a laser light scattered at 4 different angles. It does not use erythrocyte lysis and thus can, in theory, be used on avian blood. However, a previous study in domestic chickens (*Gallus gallus*) suggested that CD can only provide an accurate estimation of granulocyte counts.[7]

The objectives of this study were (1) to compare WBC counts obtained using CD, PB, and EBS; (2) to compare the differential leukocyte counts obtained with CD to the differential leukocyte counts obtained with EBS; (3) to evaluate interobserver and intraobserver variations in estimated total leukocyte counts and differential leukocyte counts from EBS; and (4) to evaluate the repeatability of the measures obtained with each method. We hypothesized that EBS could provide a clinically useful and repeatable estimation of leukocyte counts and the differential leukocyte counts. We also hypothesized that CD would not prove to be an appropriate replacement for manual diagnostic testing methods used for complete blood cell counts in avian hematology.

MATERIALS AND METHODS

All procedures were approved by the *Faculté de Médecine Vétérinaire* Animal Care and Use Committee under the auspices of the Canadian Council on Animal Care. Blood was collected from 25 birds admitted to the Exotic Animal Clinic and the Raptor Rehabilitation Clinic of the Université de Montréal's Faculté de médecine vétérinaire. Birds of variable health status were included in this study. Sixteen different species were represented. Venipuncture was performed in the ulnar vein for the Falconiformes (n = 5), Strigiformes (n = 8), and Columbiformes (n = 1); in the jugular vein for the Psittaciformes (n = 7); and in the medial metatarsal vein for the Anseriformes (n = 4). Blood was collected using a 1-mL syringe and a 25-G needle. A blood smear was immediately prepared with the fresh blood using a standard 2-slide wedge technique with Propper Bev-l-edge slides (Propper Manufacturing Company, Long Island City, NY, USA).[13] The remaining blood was placed in heparinized microtubes (Starstedt AG & Co, Nümbrecht, Germany) and inverted 10 times to ensure adequate mixing and anticoagulation. The tubes were then stored at 4°C (39.2°F) until an analysis of the samples was performed.

Within 5 minutes of venipuncture, 2 micro-hematocrits were measured via centrifugation (Statspin Technologies, Westwood, MA, USA), and the mean hematocrit value was calculated. All other analyses were performed within 24 hours of sampling. Complete leukogram results were obtained on each sample using CD. The analysis was repeated 5 times for 3 samples to evaluate the repeatability of the CD method. For each sample, 5 heterophil and eosinophil (H&E) counts were obtained from the heparinized blood using a commercial PB method (Unopette Eosinophil 5877, Becton-Dickinson, Franklin Lakes, NJ, USA) and a Neubauer hemocytometer (Prosource Scientific, Ottawa, Ontario, Canada). All H&E counts were performed by the same observer using the same microscope (Leica DMLS I/03 with objective Leica 40 × N Plan, numerical aperture 0.65, Leica Microsystems, Wetzlar, Germany). Total H&E count/μL (*Tot H&E*) was then calculated using the following equation[14]:

$$Tot\,H\,\&\,E = \frac{H\,\&\,E}{2} * 32 * \frac{10}{9}$$

where *H&E* is the number of heterophils and eosinophils counted in both of the hemocytometer's chambers. Total leukocyte count (*Tot Leuk*) was then calculated with the following equation[5]:

$$Tot\,Leuk = \frac{Tot\,H\,\&\,E * 100}{\%heterophils + \%eosinophils}$$

where *% heterophils + % eosinophils* is the percentage of these 2 leukocyte types determined by manual differentiation as described below.

All 25 blood smears were stained with an automated Wright's stain (Wescor 7120 Hematology Slide Stainer, Wescor, Logan, UT, USA). The 4 observers (A: zoological medicine intern; B: zoological medicine clinician; C: zoological medicine professor; D: clinical pathology professor) determined a complete leukogram (total estimated leukocyte counts and differentials) once for each smear. Blood smear estimation was performed an additional 4 times by observer A on each smear. Before the beginning of the study, all observers participated in a group training session to standardize the blood smear evaluation technique, in which they agreed on the cellular density of the fields to be selected and the criteria for leukocyte identification. Throughout the study, the observers were not allowed to communicate their results and were blinded to the species, origin, and history of the birds.

The total estimated WBC count (*EWBC*) was calculated on 10 and 20 microscope fields at 40× using the following equation[14]:

$$EWBC = \frac{N\ leukocytes}{N\ fields} * 2$$

where *N leukocytes* is the number of leukocytes counted on all the selected fields and *2* is the estimation factor commonly described in the literature.[14–16] To avoid errors with very anemic or polycythemic birds, *EWBC* was corrected (*cEWBC*) with the following equation[5]:

$$cEWBC = EWBC * \frac{Observed\ PCV}{45}$$

where *Observed PCV* is the micro-hematocrit measured for each bird, and *45* is the normal mean packed cell volume (PCV) value in birds.[5] Finally, differential leukocyte counts were performed by determining the number of each leukocyte type (heterophils, eosinophils, lymphocytes, and monocytes) for 100 cells on 100 × microscope fields. All results are given in international units ($\times 10^9$/L).

Coefficients of variation (CV), calculated as the SD divided by the mean and expressed as a percentage, were used to assess interobserver and intraobserver variability and to determine method precision. The Kappa coefficient was used to assess interobserver agreement using 3 categories of estimated WBC ($<5 \times 10^9$/L, $\geq 5 \times 10^9$/L to $\leq 20 \times 10^9$/L, and $>20 \times 10^9$/L). These categories reflected leukopenia, reference interval, and leukocytosis. Intraclass correlation coefficients (ICC) were calculated using a mixed linear model to evaluate the agreement of measurements obtained from 10 and 20 fields by 1 of the observers and to evaluate the agreement of measurements from 10 and 20 fields across the different observers. The ICC values >90% were considered to reflect excellent agreement. Further, this determined that there was no apparent benefit to evaluating 20 fields instead of 10; therefore, results obtained from 10 fields were used to evaluate agreement between methods. The mean WBC estimates obtained from all observers with EBS and CD were compared with PB as the reference standard. This was done with a modified Bland-Altman analysis for repeated measures and calculating ICCs for WBC counts and differentials between methods. Statistical analyses were conducted using statistical analysis software (SAS 9.1, SAS Institute Inc, Cary, NC, USA). The threshold for statistical significance of results was $P < 0.05$.

Table 1. Kappa coefficient for the assessment of interobserver agreement for 4 observers using 2 estimation factors, including standard (2) and corrected with the phloxine B stain method as a reference standard (1.5).

	Observers				
Estimation factor	A and B	A and C	A and D	B and C	C and D
Standard (2)	0.70	0.60	0.76	0.79	0.74
Corrected (1.5)	0.92	0.70	0.83	0.78	0.85

RESULTS

Two smears were excluded from the study due to defective staining; thus, statistical results were calculated from samples obtained from 23 birds.

EBS method evaluation

The interobserver CV between 4 observers for the estimated WBC from EBS counts on 23 smears was 18.5%. Intraobserver CV from EBS counts was 11.2%. The median CVs for the percentages of cell types reported by the 4 observers are as follows: heterophils (12.7%), eosinophils (138.3%), lymphocytes (50.1%), and monocytes (120.4%). As such, substantial interobserver variability was noted for eosinophils and monocytes and, to a lesser extent, lymphocytes, while superior agreement was noted for heterophil counts. This variability was greatly reduced when H&E were grouped together and compared separately from the mononuclear cells (lymphocytes and monocytes), resulting in a CV of 11.5% and 13.7%, respectively. Moreover, this variability was also reduced when observer A, the least experienced observer, was excluded from the analysis.

Interobserver agreement based on the 3 WBC categories (leukopenia $<5 \times 10^9$/L, reference interval $\geq 5 \times 10^9$/L to $\leq 20 \times 10^9$/L, and leukocytosis $>20 \times 10^9$/L) was good (Kappa: 0.60–0.79). Observers classified smears in the same categories in 65–83% of cases. Kappa values for the assessment of interobserver agreement are summarized in Table 1. When a reference

Table 2. The number of falsely classified leukocyte counts when the average total white blood cell count is determined from an estimate from blood smear on 10 and 20 microscope fields and phloxine B is used as the reference method. Correlation between estimated total white blood cell counts using an estimate from blood smear on 10 and 20 fields was excellent (intraclass correlation coefficient of 94.9–99.2%). A single individual had a different classification based on the number of fields used. The blood samples were collected from 23 birds (Falconiformes [n = 5], Strigiformes [n = 8], Columbiformes [n = 1], Psittaciformes [n = 7], and Anseriformes [n = 4]).

	Leukopenia ($<5 \times 10^9$/L)	Reference interval ($\geq 5 \times 10^9$/L to $\leq 20 \times 10^9$/L)	Leukocytosis ($>20 \times 10^9$/L)
Number of samples classified with PB	3	16	4
False classification with EBS using 10 fields	0	2	1
False classification with EBS using 20 fields	0	3	1

Abbreviations: Pb, phloxine B; EBS, estimate from blood smear.

standard is available, the estimation factor included in the formula used for the EBS method can be calculated with the following formula[17]:

$$\frac{\text{Mean total WBC reference standard}}{\text{Mean number leukocytes per field}}$$

If we consider PB as the reference method, as suggested by some authors, the estimation factor calculated using this formula is 1.5.[5,8,9] The Kappa analysis was repeated with the results obtained with this new estimation factor (Table 1). Kappa values improved and observers classified smears in the same categories in 83–96% of cases.

When the evaluation of EBS on 10 and 20 fields was compared regarding the classification of average WBC counts as leukopenic, within the reference range, or leukocytosis, only 1 of 23 birds was classified differently based on the number of fields used. No difference in the classification of results obtained with EBS on 10 or 20 fields was seen in the birds with more severe leukopenia or leukocytosis. These results are compared with those obtained with PB as a reference method in Table 2.

PB method evaluation

For the PB method, the intraobserver CV was 13.4% for the 5 H&E counts obtained from each smear by a single observer. The interobserver CV was 10.5% for total leucocyte counts calculated using the percentage of H&Es obtained by manual differentiation by each observer.

CD method evaluation

The calculated CV for the CD method was 5.9%. This was evaluated using 3 blood smears that were each measured 5 times with this method.

Method comparisons

The ICCs for different leukogram determination methods are summarized in Table 3. Excellent agreement (ICC >90%) was found between EBS counts obtained from 10 and 20 fields, across the 4 observers for WBC counts with EBS, WBC counts with PB, and H&E counts with PB. Good agreement (ICC between 80% and 90%) was found for WBC counts between CD and PB, and moderate agreement (ICC between 60% and 80%) was found for WBC counts between EBS and PB and between EBS and CD. However, poor agreement (ICC <25%) was found between EBS and CD for individual white blood cell types (heterophils, eosinophils, lymphocytes, and monocytes). Differences between WBC and H&E counts obtained between the different methods are represented in Figure 1.

The modified Bland-Altman analysis for repeated measures comparing CD and PB showed that 95% of

Table 3. Intraclass correlation coefficients obtained by the comparison of different variables within and between the 3 leukogram determination methods. The blood samples were collected from 23 birds (Falconiformes [n = 5], Strigiformes [n = 8], Columbiformes [n = 1], Psittaciformes [n = 7], and Anseriformes [n = 4]).

Variables	ICC, %
EBS: 10 and 20 fields	94.9–99.2
EBS: 10 fields	
WBC	92.9
PB	
WBC	96.6
Heterophils and eosinophils	99.9
EBS and PB	
WBC	69.5–81.3
EBS and CD	
WBC	63.9–80
Heterophils	0–4.3
Eosinophils	0
Lymphocytes	0–12
Monocytes	0–23.4
CD and PB	
WBC	85.9–91.5

Abbreviations: CD, Cell Dyn 3500; EBS, blood smear; ICC, intraclass correlation coefficients; PB, phloxine B stain method; WBC, total white blood cell count.

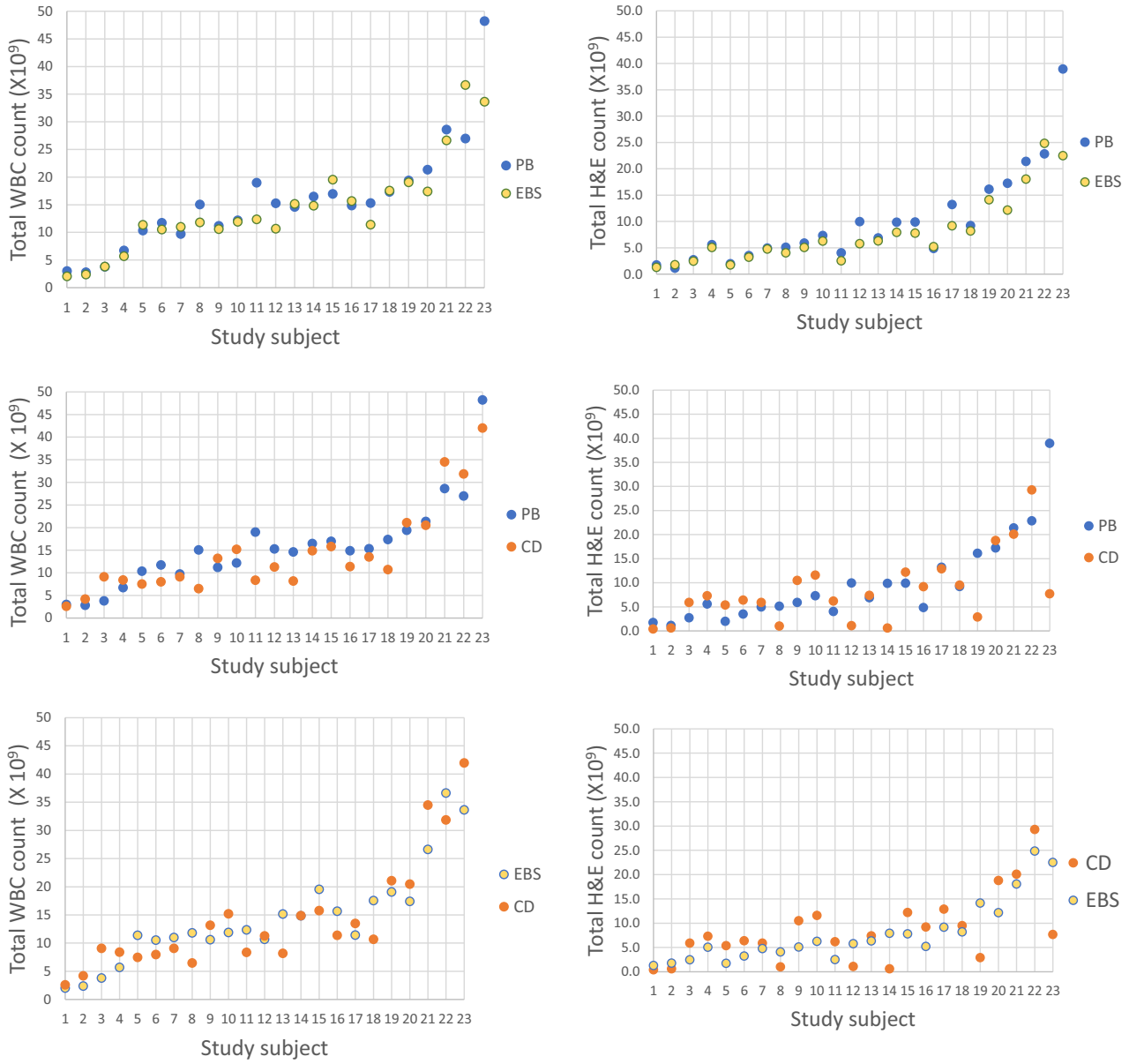

Figure 1. Comparison of total white blood cell (WBC) counts and total heterophil and eosinophil (H&E) counts between the 3 leukogram determination methods (EBS: Estimate from blood smear; PB: Phloxine B stain method; CD: Cell Dyn 3500). The blood samples were collected from 23 birds (Falconiformes [n = 5], Strigiformes [n = 8], Columbiformes [n = 1], Psittaciformes [n = 7], Anseriformes [n = 4]).

differences were included between −10.1 and 7.2 (Fig 2, Table 4). The slope (−1.4) was not significantly different from 0 ($P = 0.13$), and the intercept (−1.72) was not significantly different from 0 ($P = 0.35$), indicating that there is no systematic bias between the 2 measurements.

Finally, the modified Bland-Altman analysis for repeated measures comparing EBS results with a 1.5 estimation factor and PB showed that 95% of differences were included between −9.0 and 8.1 (Fig 3, Table 4). The linear regression between the paired differences and the mean values revealed that the slope (−0.62) was not significantly different from 0 ($P = 0.52$). The intercept (0.49) was not significantly different from 0 ($P = 0.79$), indicating that there were no systematic or proportional biases between the 2 measurements (Fig 3, Table 4).

DISCUSSION

The comparison of the estimated WBC results between 10 and 20 fields showed excellent correlation, indicating that there was no benefit to evaluating 20 fields instead of 10 to determine the estimated WBC on a blood smear.

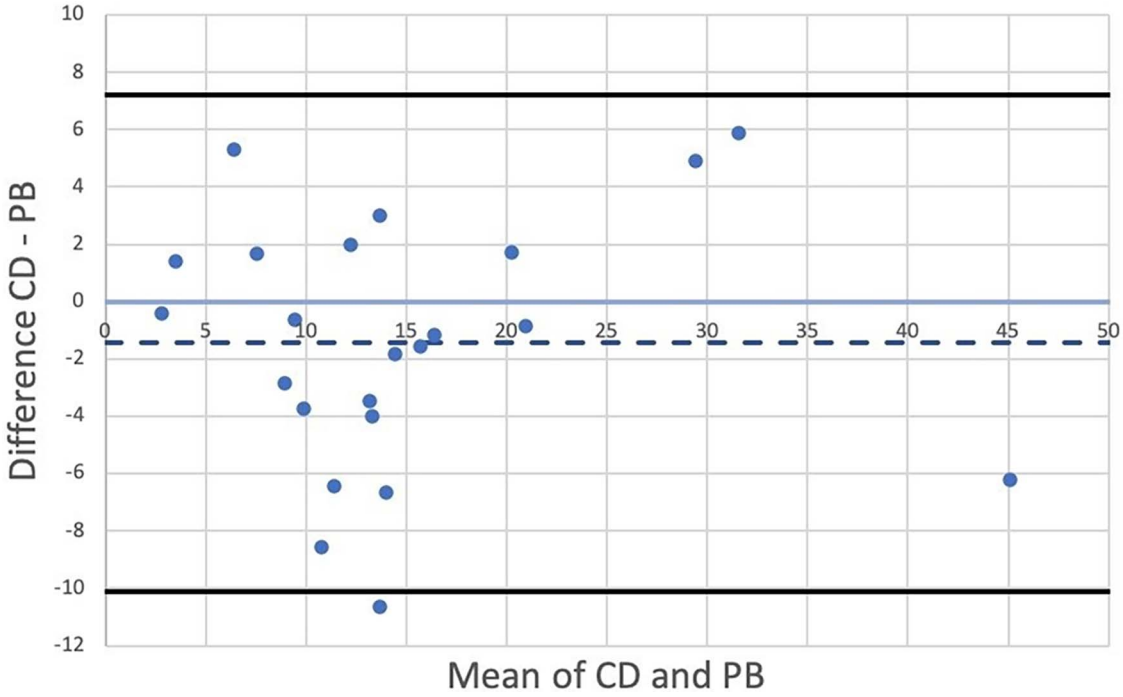

Figure 2. Bland-Altman plot of total white blood cell counts using the following 2 different methodologies: a commercial phloxine B stain method (PB) and an automated analyzer using impedance and flow cytometry (CD). The blood samples were collected from 23 birds (Falconiformes [n = 5], Strigiformes [n = 8], Columbiformes [n = 1], Psittaciformes [n = 7], and Anseriformes [n = 4]). The solid blue line indicates perfect agreement between methods (0), the solid black lines indicate the upper and lower 95% limits of agreement, and the dashed blue line indicates the mean difference between methods (−1.45). Ninety-five percent of differences were included between −10.1 and 7.2. The slope (−1.4) was not significantly different from 0 ($P = 0.13$), indicating the bias did not change systematically as the mean values increased.

The CV for the WBC was lower with PB (10.5%) than with EBS (18.5%), indicating a superior performance with this method, as suggested by several authors.[7–9] However, the statistical analysis for estimated WBC using EBS showed that there was generally very good agreement between observers and that they classified smears in the same categories (leukopenia, reference interval, or leukocytosis) in more than 83% of cases. Disagreement in classification occurred when results obtained by some observers were at the high end of the reference interval ($\geq 17 \times 10^9$/L to $\leq 20 \times 10^9$/L) and at the low end of the leukocytosis classification ($\geq 20 \times 10^9$/L to $\leq 22 \times 10^9$/L) for the other observers. No disagreement was noted for the leukopenic classification. Therefore, this method appears clinically useful for determining the total estimated WBC across several avian species. Similar findings have been reported for EBS use in cockatiels (*Nymphicus hollandicus*).[18] The PB method in this study was performed through the use of the commercially available unopette eosinophil kit. Although it was available for the duration of this study, it was discontinued during the preparation of this manuscript. A commercial alternative is now available, The Avian Leukopet (Vetlab Supply, Palmetto Bay, FL, USA).[19]

For EBS, the intraobserver CV (11.2%) was lower than the interobserver CV (18.5%), indicating that the

Table 4. Agreement parameters (Bland-Altman) for total white blood cell count determined using 3 leukogram determination methods: estimate from a blood smear, phloxine B stain method, and Cell Dyn 3500. The blood samples were collected from 23 birds (Falconiformes [n = 5], Strigiformes [n = 8], Columbiformes [n = 1], Psittaciformes [n = 7], and Anseriformes [n = 4]).

Method 1	Method 2	Slope (95% CI)	Slope p value	Slope t value	Intercept (95% CI)	Intercept p value	Intercept t value	Mean difference	Bland-Altman agreement
EBS	PB	−0.62 (−9.62-8.37)	0.52	−0.65	0.49 (−3.09-4.08)	0.79	0.27	−0.62	Yes
CD	PB	−1.44 (−10.10-7.22)	0.13	−1.57	−1.72 (−5.3-1.77)	0.35	−0.97	−1.45	Yes

Abbreviations: CD, Cell Dyn 3500; EBS, estimate from blood smear; CI, confidence interval PB, phloxine B stain.

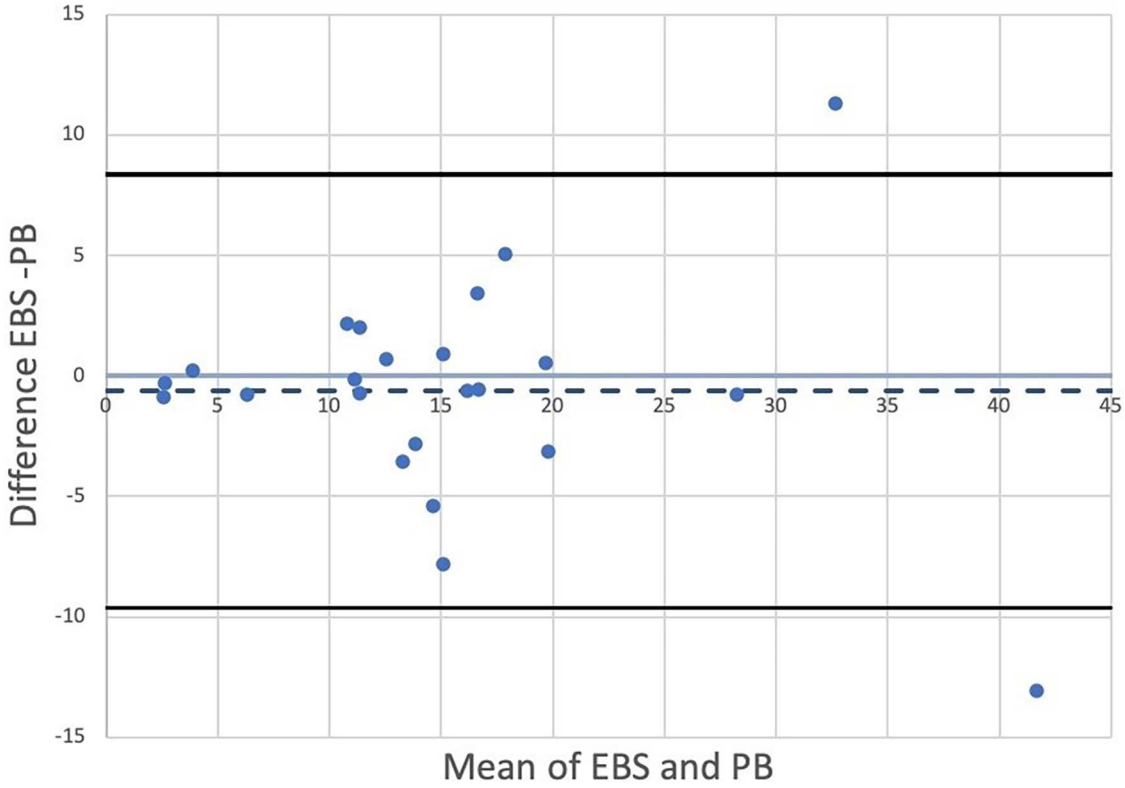

Figure 3. Bland-Altman plot of total white blood cell counts using the following 2 different methodologies: a commercial phloxine B stain method (PB) and an estimation from a blood smear (EBS). The blood samples were collected from 23 birds (Falconiformes [n = 5], Strigiformes [n = 8], Columbiformes [n = 1], Psittaciformes [n = 7], and Anseriformes [n = 4]). The solid blue line indicates perfect agreement between methods (0), the solid black lines indicate the upper and lower 95% limits of agreement, and the dashed blue line indicates the mean difference between methods (−0.62). Ninety-five percent of the differences were included between −9.6 and 8.4. The slope (−0.62) was not significantly different from 0 (P = 0.52), indicating the bias did not change systematically as the mean values increased.

repeatability of this method is good, which is important when assessing the evolution of a leukogram over time in a sick bird. Based on these results, the same observer should perform repeated leukograms on an individual.

Analyses for leukocyte differential counts showed that variations were more important for eosinophils, lymphocytes, and monocytes than heterophils. Confusion occurred within mononuclear cell types (lymphocytes and monocytes) and granulocyte cell types (H&E). Hypotheses accounting for the confusion between H&E include defective coloration and the fact that these 2 types of granulocytes are very similar in some avian species.[2] Possible causes for the confusion between lymphocytes and monocytes are their similarity when lymphocytes are large and clear and a lack of agreement on the criterion for recognition of cells. Monocytes and lymphocytes may also appear similar to thrombocytes in avian blood. Additionally, the variations observed in cell identification were reduced when observer A, who was the least experienced, was excluded from the analysis. This suggests that training and experience are important for consistency in cell recognition.

A previous study has suggested that CD was not reliable for leukocyte differentials but was of clinical usefulness for total granulocyte counts.[7] The CD was reported as unable to differentiate between H&E, and it was suggested that the analyzer may also have difficulty identifying lymphocytes from thrombocytes or immature erythrocytes. This study obtained very low ICC between CD and EBS for H&E, corroborating that the analyzer could not properly identify granulocyte cell types. Comparisons between CD and the other methods showed that this analyzer was not reliable for leukocyte differentials or H&E counts but had good agreement with PB for total WBC counts. The reason why the results obtained in this study do not agree with those obtained in the prior study is unclear. One possible explanation may lie in the high number of different species used in this study, whereas the previous study was only performed on chickens. Avian blood cell size may vary widely across species.[20,21] Additionally, for leukocytes, the presence of stressors such as corticosterone or lipopolysaccharides found in bacterial membranes may also result in

cell size variation and increase the presence of secretory granules within the cells, as reported in chickens.[22] Stress or disease could, therefore, affect granulocyte appearance and size and their ability to be correctly classified by an automated analyzer. Differences in leukocyte morphology among the species in this study and the variable health status of individuals may thus have affected results obtained with CD.

A major limitation of this study lies in its field of interest, avian hematology. As there is no reference standard for the quantification of white blood cells in avian species, the authors emphasize the value of establishing reference values within a laboratory or a hospital so that results may be compared with those obtained using the same techniques and equipment.

Although less precise than PB, EBS is a clinically useful method that requires no specialized laboratory equipment and is time efficient for the estimation of WBC. Furthermore, it is the only way to obtain a simultaneous leukocyte differential and WBC without the additional step of hemocytometer counting required with PB. However, this study shows that the estimation factor to include in the formula for this method must account for the field covered by each 40× objective to avoid an overestimation of the total WBC. This method also requires staff within a clinic or laboratory to standardize the steps needed for the WBC estimation and leukocyte differential and establish criteria for cell recognition. The CD does not seem adapted to avian hematology for leukocyte differential counts.

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