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Authors: Godfrey, Ralph D., Fedynich, Alan M., and Pence, Danny B.

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QUANTIFICATION OF HEMATOZOA IN BLOOD SMEARS

Ralph D. Godfrey, Jr.,[1] Alan M. Fedynich,[1] and Danny B. Pence[2,3]

[1] Department of Range and Wildlife Management, Texas Tech University, Lubbock, Texas 79409, USA
[2] Department of Pathology, Texas Tech University Health Sciences Center, Lubbock, Texas 79430, USA
[3] Author to whom reprint requests should be addressed

ABSTRACT: Ten thin blood smears from mourning doves (*Zenaida macroura*) infected with *Haemoproteus maccallumi* were examined by each of two observers using identical techniques and microscopy in an attempt to delineate the factors necessary to provide an accurate estimate of the number of parasites/*n* erythrocytes. The number of erythrocytes examined must be actually counted, not estimated from extrapolated partial counts or from the number of fields of view examined. Doubling the number of erythrocytes counted (1) decreased the overdispersed frequency distribution patterns in only 25% of the replicate counts for numbers of *H. maccallumi*/100 erythrocytes for a series of 2,000 versus 4,000 erythrocytes counted; and (2) did not significantly increase the accuracy for determining parasite intensities. Thus, the number of erythrocytes that must be counted to determine parasite intensities could be considerably reduced from the 10,000 or 20,000 estimated for most studies, and still provide an accurate determination of the number of parasites/*n* erythrocytes in datasets collected from hosts with moderate to high levels of parasitemia. This resulted in a decreased amount of time expended by the observer on each blood smear examined. With two equivalently trained individuals, differences between observers examining the same blood smears were minimal. This study suggests an approach by which a more standardized methodology for quantifying blood parasite intensities could be developed.

Key words: Quantification, avian Hematozoa, thin blood smears, mourning doves, *Zenaida macroura*, *Haemoproteus maccallumi*.

INTRODUCTION

Because of the inherent difficulties involved in the quantification of Hematozoa in thin blood smears, most previous studies have reported only data on prevalence. However, certain studies have emphasized the importance of determining the intensities of Hematozoa, and have utilized various techniques for quantifying parasites in thin blood smears (Garnham, 1966; Coatney et al., 1971; Barnard and Bair, 1986; Bromwich and Schall, 1986; van Riper et al., 1986). These techniques are only partially quantitative (include qualitative elements), lack an adequate estimate of error, and are not standardized so that interpretation of data between different studies is possible. Difficulties in quantification of Hematozoa in blood smears may result from: (1) heterogeneity of distribution of erythrocytes in blood smears, (2) heterogeneity of distribution of parasitized erythrocytes in blood smears, (3) estimates of the number of erythrocytes/field of view, (4) number of erythrocytes that must be counted to give an adequate estimate of parasite intensity, (5) number of fields of view examined, (6) length of time required by an observer to examine a blood smear, and (7) variation between observers examining the same blood smear. The present study addresses these variables and presents an approach by which a more standardized technique for quantification of Hematozoa in thin blood smears could be developed.

MATERIALS AND METHODS

Thin blood smears were prepared from the heart blood of mourning doves (*Zenaida macroura*) collected by shooting during 1 to 2 September 1985 from one locality in Foard County, Texas. Blood smears were prepared immediately following death of the host, allowed to air dry, stored in slide boxes, and transported to the laboratory where they were stained 10 min with phosphate-buffered Giemsa (pH 7.2) after fixation in 100% methanol for 1 min. Ten thin blood smears each from 10 different doves infected with *Haemoproteus maccallumi* were selected for examination based on adequate staining and uniformity of the smear. These were examined at 1,000× oil immersion by each of two observers (AMF and RDG) using identical

light microscopes (A. O. Microstar, American Optical Corporation, Buffalo, New York 14240, USA) each equipped with a Miller ocular disc (A. O. K1282, American Optical Corporation).

To determine if the number of erythrocytes within a particular field of view could be estimated by extrapolation of partial area counts, the number of erythrocytes were counted in the small inner square (0.25 mm) and large square (0.75 mm) of the Miller disc. Erythrocytes in both the small inner square and the large square were counted from 10 separate fields of view by each observer for each of the 10 blood smears. Erythrocytes intersecting the boundaries on two adjacent sides of each of the squares were counted. Erythrocyte counts in the small inner square were multiplied by a factor of nine to provide an estimate of the total number of erythrocytes present within the area of the large square. The estimated number of erythrocytes within the area of the large square were compared with the actual number counted using a *t*-test (PROC TTEST, Statistical Analysis Systems, 5th edition, SAS Institute Inc., Cary, North Carolina 27511, USA). Chi-square analysis tested for differences in erythrocyte counts within the large square of the Miller disc for 10 replicates on each blood smear as examined by each individual observer.

In order to determine if hematozoan intensities varied with the number of erythrocytes counted, the number of *H. maccallumi*/100 erythrocytes were counted in replicates of 20 and 40 (2,000 and 4,000 erythrocytes, respectively) by both observers. For these data each replicate represented one or more different fields of view delineated by the large square of the Miller disc (>20 and >40 fields of view, respectively). These data did not meet the assumptions for a normal distribution (PROC UNIVARIATE, SAS). Thus, the rank transformation procedure of Conover and Iman (1981) was applied to the dataset for subsequent analyses (PROC RANK, SAS). Chi-square analysis was used to determine if the variance was significantly greater than the mean (for the frequency distributions of the 10 replicates of 20 and 40) for each blood smear examined by each observer, and the value of k (Bliss and Fisher, 1953) measured the relative degree of overdispersion of parasite abundances. Chi-square analysis was used to determine homogeneity of values of k between the 20 and 40 replicates (expected value = $\bar{x}$ of the 20 + 40 replicate series) in each blood smear examined by each of the two observers. One-way ANOVA tested for significant differences in the mean number of *H. maccallumi*/100 erythrocytes between the 20 and 40 replicates and between individual observers (PROC GLM, SAS).

Two of 10 blood smears used for comparing differences in mean Hematozoa/100 erythrocytes in the 20 and 40 replicate treatments were selected randomly by each observer and the number of Hematozoa/100 erythrocytes was recorded for 200 replicates. Since these data also indicated an other than normal distribution, they were rank transformed prior to subsequent analysis. The three sets of replicate series totaling 2,000, 4,000, and 20,000 erythrocytes counted, respectively, were tested for significant differences between numbers of *H. maccallumi*/100 erythrocytes using one-way ANOVA (PROC GLM, SAS).

Definitions of prevalence, intensity, and mean intensity followed the terminology of Margolis et al. (1982). The terms significant or significantly refer to statistical significance at $P \leq 0.05$. Representative slides of *H. maccallumi* examined in this study are deposited in the U.S. National Parasite Collection, Beltsville, Maryland 20705, USA (No. 79666) and the International Reference Centre for Avian Haematozoa, Memorial University, St. John's, Newfoundland, Canada A1B 3X9 (Nos. 97895a, b).

RESULTS

There were significant differences between extrapolated versus actual counts of erythrocytes in 19 of 20 blood smears examined by two observers (Table 1). Additionally, comparisons by total chi-square analyses of the mean erythrocyte counts (expected values are the means generated from the 10 replicates for each of 10 blood smears examined by the two observers) versus the actual number of erythrocytes counted in each individual replicate (observed values) indicated significant differences in 14 of 20 (70%) comparisons.

The values of k, an inverse measure of overdispersion, ranged from 1.3 to 21.4 ($\bar{x}$ = 6.0) and 1.1 to 39.0 ($\bar{x}$ = 9.2) for the replicates in the 20 and 40 replicate series, respectively. For counts of the number of parasites/100 erythrocytes on each blood smear used for subsequent analyses of the 20 and 40 replicate series, the variance was not significantly larger than the mean. The values of k were sufficiently high to indicate a lack of overdispersion of parasitized erythrocytes on any of the 10 blood smears examined by either observer. This indi-

TABLE 1. Mean values of estimated versus actual numbers of erythrocytes from 10 replicates of 10 thin blood smears collected from mourning doves in Foard County, Texas.

Observer	Slide	Number of erythrocytes		t	P
		Estimated[a] $\bar{x}$ ± SE[c]	Actual[b] $\bar{x}$ ± SE		
1	1	64.8 ± 7.5	47.6 ± 5.3	5.02	0.0007*
	2	88.2 ± 7.0	59.0 ± 5.3	10.09	0.0001*
	3	114.3 ± 6.3	75.8 ± 3.5	9.95	0.0001*
	4	91.8 ± 5.3	62.7 ± 3.4	7.23	0.0001*
	5	90.0 ± 8.9	60.7 ± 4.1	4.43	0.0017*
	6	108.9 ± 6.8	74.2 ± 4.4	8.48	0.0001*
	7	94.5 ± 6.3	72.7 ± 5.8	7.28	0.0001*
	8	93.6 ± 6.0	67.1 ± 2.9	6.62	0.0001*
	9	90.9 ± 4.5	58.9 ± 1.1	6.78	0.0001*
	10	102.6 ± 7.1	73.3 ± 4.0	6.02	0.0002*
2	1	72.0 ± 6.8	49.2 ± 5.5	8.37	0.0001*
	2	112.5 ± 10.4	84.5 ± 5.3	3.65	0.0053*
	3	81.0 ± 7.7	63.2 ± 3.2	2.00	0.0761
	4	72.0 ± 8.6	50.8 ± 4.1	3.17	0.0113*
	5	98.1 ± 8.2	67.8 ± 3.6	4.74	0.0011*
	6	73.8 ± 4.2	55.2 ± 3.3	4.38	0.0018*
	7	81.9 ± 9.9	53.0 ± 4.7	4.14	0.0025*
	8	82.8 ± 10.1	59.6 ± 6.3	3.32	0.0090*
	9	89.1 ± 3.4	62.6 ± 2.1	6.63	0.0001*
	10	90.0 ± 6.7	54.6 ± 5.5	7.55	0.0001*

[a] Small inner square of the Miller disc for counts of erythrocytes × 9 as an estimate of number of erythrocytes in the large square.
[b] Actual counts of erythrocytes in the large square of the Miller disc.
[c] Mean ± standard error for number of Hematozoa/100 erythrocytes.
* Significant at $P \leq 0.05$.

cated a relatively uniform pattern of distribution of parasitized erythrocytes on each slide. Comparisons of the 20 and 40 replicates indicated that the variance was not significantly different from the mean for the frequency distribution based on 10 blood smears examined by each observer. The values of k derived from data generated by the two observers examining 10 blood smears each were homogeneous between the 20 and 40 replicates in 15 of 20 (75%) comparisons. In the 25% of these comparisons that were significantly different, the heterogeneity was accounted for by increased values of k in the 40 versus 20 replicate series.

Nineteen of 20 comparisons for the mean number of *H. maccallumi*/100 erythrocytes between the 20 versus 40 replicate series were not significantly different (Table 2). Additionally, there were no significant differences in mean parasite counts between individual observers in the 20 replicate series (n = 10 replicates/observer), but mean parasite counts for the 40 replicates indicated two of 10 significant differences between observers (F_1 = 5.8, P_1 = 0.018; F_2 = 4.6, P_2 = 0.034).

The variance was not significantly greater than the mean for the frequency distribution of *H. maccallumi* between the 20, 40, and 200 replicate series based on two blood smears examined by each of the two observers. The values of k were 1.6–21.4 ($\bar{x}$ = 9.1), 2.6–39.0 ($\bar{x}$ = 13.9), and 4.3–48.1 ($\bar{x}$ = 25.9) for 20, 40, and 200 replicates, respectively. Comparison of k values across the three replicate series indicated significant differences (heterogeneity) for each of the four smears (k = 10.4, 73.7, 8.4 and 52.3, respectively). The ranked means were not significantly dif-

TABLE 2. Mean number of *Haemoproteus maccallumi*/100 erythrocytes for replicates of 2,000 and 4,000 erythrocytes counted by two observers in blood smears collected from mourning doves in Foard County, Texas.

Observer	Slide	Number of replicates[a] 20 ($\bar{x}$ ± SE[b])	40 ($\bar{x}$ ± SE)	F	P
1	1	3.5 ± 0.3	3.4 ± 0.2	0.19	0.661
	2	0.5 ± 0.2	0.6 ± 0.1	0.03	0.873
	3	0.6 ± 0.2	0.5 ± 0.1	0.04	0.852
	4	0.2 ± 0.1	0.3 ± 0.1	0.39	0.535
	5	0.8 ± 0.2	1.0 ± 0.2	0.56	0.458
	6	0.7 ± 0.2	1.0 ± 0.1	1.84	0.181
	7	0.8 ± 0.2	0.5 ± 0.1	0.53	0.468
	8	1.6 ± 0.2	1.0 ± 0.1	5.67	0.021*
	9	0.9 ± 0.2	0.9 ± 0.1	0.08	0.772
	10	0.6 ± 0.2	0.6 ± 0.1	0.35	0.555
2	1	3.4 ± 0.4	4.2 ± 0.4	1.11	0.297
	2	0.6 ± 0.1	0.5 ± 0.1	0.34	0.560
	3	0.6 ± 0.1	1.0 ± 0.2	2.31	0.134
	4	0.5 ± 0.2	0.5 ± 0.1	0.00	0.985
	5	0.7 ± 0.2	0.5 ± 0.1	0.97	0.330
	6	1.0 ± 0.3	0.9 ± 0.2	0.03	0.875
	7	0.7 ± 0.2	0.3 ± 0.1	1.50	0.225
	8	1.1 ± 0.2	1.0 ± 0.1	0.40	0.530
	9	1.1 ± 0.2	1.0 ± 0.2	0.40	0.528
	10	0.7 ± 0.2	0.7 ± 0.1	0.03	0.865

[a] 100 erythrocytes counted/replicate.
[b] Mean ± standard error for number of Hematozoa/100 erythrocytes.
* Significant at $P \leq 0.05$.

ferent for the number of *H. maccallumi*/100 erythrocytes in three of the four blood smears across replicates of 20, 40, and 200 (Table 3). A protected least significant difference test indicated that the mean number of *H. maccallumi* was significantly higher in the 20 replicate versus the 40 and 200 replicate series in one of four blood smears (Table 3).

Average time required by the observer to count erythrocytes in each replicate series of 20, 40 and 200 was 35, 60 and 300 min, respectively. Time expended counting erythrocytes by each observer for the 10 blood smears examined was 350 and 600 min for the 20 and 40 replicate series, while the four blood smears examined for the 200 replicate series required 1,200 min. It required 2,150 min (36 hr) for each observer to examine all replicates in the 10 blood smears.

TABLE 3. Mean number of *Haemoproteus maccallumi*/100 erythrocytes for replicates of 2,000, 4,000, and 20,000 erythrocytes counted in four blood smears collected from mourning doves in Foard County, Texas.

Slide	Number of replicates[a] 20 ($\bar{x}$ ± SE[b])	40 ($\bar{x}$ ± SE)	200 ($\bar{x}$ ± SE)	F	P
1	0.8 ± 0.2	1.0 ± 0.2	0.6 ± 0.1	2.98	0.052
2	1.6 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	5.67	0.004*[c]
3	0.6 ± 0.1	1.0 ± 0.3	0.8 ± 0.1	1.25	0.288
4	0.5 ± 0.2	0.5 ± 0.1	0.4 ± 0.1	0.55	0.579

[a] 100 erythrocytes/replicate.
[b] Mean ± standard error for number of Hematozoa/100 erythrocytes.
[c] Least significant differences test indicates that the 20 replicate series is significantly different from the 40 and 200 replicate series.
* Significant at $P \leq 0.05$.

DISCUSSION

In an attempt to quantify Hematozoa, many studies have assumed that (1) the distribution of erythrocytes and parasitized erythrocytes are homogenous in blood smears and (2) a certain number of fields of view and/or period of time spent examining blood smears provides an accurate estimate of the number of parasites/n erythrocytes (Trainer et al., 1962; Shamis and Forrester, 1977; Williams et al. 1977; Shaw, 1978; Barnard and Bair, 1986; Bromwich and Schall, 1986; van Riper et al., 1986). Our results indicate that these methods used to estimate the total number of erythrocytes do not provide an accurate determination of the number of erythrocytes counted or the parasite intensities. Shamis and Forrester (1977) recognized the problems inherent in using these estimation techniques and indicated that the actual number of parasites reported included smear scanning and sampling errors. In this study, we found marked heterogeneity in erythrocyte counts from a defined field of view across 14 of 20 blood smears. Therefore, an accurate determi-

nation of total number of erythrocytes in many fields of view cannot be extrapolated from actual counts of a few fields of view. Likewise, a pre-established period of time for examination of blood smears is not a reliable estimate of the total number of erythrocytes examined.

The above results indicated that all erythrocytes within a field of view must be counted for an accurate determination of the total number of erythrocytes examined. This is time consuming and could amount to ≥5 hr/blood smear for erythrocyte counts of 20,000. Therefore, we examined fractional counts within fields of view to determine if they could be extrapolated as an accurate estimate for total erythrocytes/ field of view. Using the Miller disc, a standard method for estimating reticulocytes in thin blood smears (Maile, 1982), the number of erythrocytes from the extrapolated data consistently, and significantly, overestimated the actual number of erythrocytes. This may have resulted from the change in perimeter-to-area ratio (increased edge effect) as described by Wiegert (1962) and Van Dyne et al. (1963). The use of the Miller disc for reticulocyte counts has certain inherent statistical errors that predetermine the limits of significance (Maile, 1982) and this limits also its usefulness for critical determination of parasite intensities.

We next attempted to define the total erythrocyte counts necessary to obtain reliable parasite intensity data. Estimates for parasites/n erythrocytes from most studies are derived from estimates of from 10,000 to 20,000 erythrocytes examined. Depending on the parasitemia, the actual number of erythrocytes that must be examined to obtain an accurate determination of hematozoan intensities may be far less. This was the case in the present study where intensities of *H. maccallumi* in mourning doves were high (0.2–4.2/100 erythrocytes). Since the mean parasite intensity in only one of 20 blood smears examined by the two observers was significantly different in the 20 versus 40 replicate series, we concluded that doubling the number of erythrocytes examined did not appreciably increase the accuracy for determining parasite intensities in our dataset. Thus, the amount of time required for examination of individual blood smears was substantially reduced (from 60 to 35 min/blood smear). Additionally, data from 200 replicates were compared with those of the 20 and 40 replicate series. The only significant difference in mean parasite intensity between the 20 versus 40 versus 200 replicate series was in the same blood smear that showed a significant difference in the 20 versus 40 replicate series. We concluded that counting 2,000 erythrocytes was sufficient to adequately determine the mean parasite intensity for this dataset.

It appears that there can be minimal differences between equivalently trained individual observers examining the same blood smears. There were no significant differences between individual observers for the 20 replicate series and only two of 10 possible significant differences between observers in the 40 replicate series. In both replicate series there were no consistent trends in high or low parasite counts between observers. The highest values recorded for mean parasite counts varied between the two observers.

Because of the many techniques devised by individual authors for quantifying Hematozoa in blood smears, there is a need for standardization in order to compare results between studies. Further, our study emphasizes the need for vigorous statistical evaluation of overdispersion and the other variables (methods for determining the number of erythrocytes and parasites/n erythrocytes) related to quantification of Hematozoa. Thus, we offer a number of recommendations toward establishing such a standardized technique.

Because of differences between peripheral versus visceral concentrations of certain blood parasites (Couch, 1952; Robinson, 1954), and for consistency of

experimental design, it is recommended that the collection site for obtaining blood samples from the host should be consistent across all individuals in a particular study. Apparently, this has been recognized in certain studies (Farmer, 1960; Loven et al., 1980; Bennett et al., 1982), while others have pooled samples from a number of host collection sites (Herman, 1951; Roslien et al., 1962; Gutierrez, 1973; Bennett et al., 1975).

Blood smears in certain studies were obtained from hosts collected by trapping, shooting, carcasses, etc. (Herman, 1951; Bennett et al., 1974; Greiner, 1975; Shamis and Forrester, 1977). A single collection method is more likely to sample hosts in similar physiological condition, versus the use of multiple collection techniques (Weatherhead and Greenwood, 1981; Greenwood et al., 1986). Therefore, because there may be differences in concentrations and/or localization of parasitized erythrocytes contingent on host condition at the time of collection (van Riper et al., 1986), we recommend that investigators adhere to a consistent method of host collection during a particular study, or that analyses be performed to test for differences prior to pooling data.

Clotting and hemocentration of blood following death of a host rapidly precludes preparation of adequate blood smears. Also, the effect of postmortem changes on Hematozoa has not been determined. Thus, determination of parasite intensities from dead hosts can have errors of unknown magnitude. It is recommended that blood smears be prepared from hosts that are alive or only immediately following their death.

Certainly, there must be an adequate number of blood smears examined per host individual and these should be completely scanned to determine the presence or absence of parasitemia in a particular host individual. More than one blood smear/host should be examined since not all smears may have parasites (O'Meara, 1956), especially in cases of low parasitemia. Each blood smear should be thoroughly scanned because there may be an uneven distribution of parasitized erythrocytes in the blood smear. Also, this will provide a realistic estimate of the numbers of erythrocytes that need to be counted for an accurate determination of parasite intensities. Although actual counts of erythrocytes must be performed and erythrocyte counts should be realistic in terms of the amount of time expended by the observer, the actual number of erythrocytes that should be quantified to provide an accurate estimate of parasite intensities in a particular host population may vary depending on the level of parasitemia. For studies with low parasite intensities it may be necessary to count greater numbers of erythrocytes, therefore increasing the time required by the observer. Although consistent results for mean parasite counts were obtained by two individual observers in our study, it is recommended that observers be adequately trained and that potential differences be determined prior to pooling data from multiple observers.

Our study examined a single species of the genus *Haemoproteus*, but many surveys deal with multiple infections of Hematozoa. We suggest that many of the above recommendations are applicable also to these studies. However, some Hematozoa such as *Leucocytozoon* spp. and certain hemoproteids that enlarge the host cells may tend to concentrate in the tails and along the edges of blood smears. Also, in intense parasitemias the parasitized erythrocytes may concentrate in the tails of smears, and intense parasitemias in some species such as *Plasmodium falciparum* tend to change the fluid dynamics of the blood (Coatney et al., 1971). This further emphasizes the necessity of (1) initial scanning in order to select representative areas on the blood smear, including the tails, for estimating parasite intensities, (2) evaluating the distribution patterns of parasitized erythrocytes on blood smears and (3)

using appropriate transformation procedures prior to further statistical comparisons of parasite intensities. The rank transformation procedure of Conover and Iman (1981) should be particularly appropriate when comparing overdispersed parasite intensities across blood smears within and between host populations. When multiple infections of hematozoan species occur, parasite intensities should be quantified independently.

Our study provides a tentative protocol for accurately quantifying Hematozoa in thin blood smears. This is suggested as an initial point from which a more standardized methodology could be developed that would permit comparison of parasite intensities between studies.

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