ACTA
AMAZONICA

Methodological limitations of counting total leukocytes and thrombocytes in reptiles (Amazon turtle, *Podocnemis expansa*): an analysis and discussion

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ABSTRACT

The aim of this paper is to compare three different methods for counting white blood cells [WBC] (Natt and Herrick method, estimation with 1,000 and 2,000 erythrocytes) and three methods for counting total thrombocytes [TT] (Wojtaszek method, estimation with 1,000 and 2,000 erythrocytes) in a South American freshwater turtle species, *Podocnemis expansa*, Schweigger 1812 (Reptilia, Pelomedusidae). Direct WBC counts using the Natt and Herrick method showed limitations, which are discussed here. The WBC and TT counts using 1,000 erythrocytes from blood smears are not recommended for Amazon turtles nor other reptilian species, since wide variation in counts can be observed. Estimation methods for determining WBC and TT based on 2,000 erythrocytes of blood smears were most acceptable because they allow a differentiation between leukocytes and thrombocytes and also had a smaller variation. The methods investigated here for the Amazon turtle, which have been widely used in other reptile species, provided evidence that the most acceptable method is not that of using diluted stains and a hemocytometer.

KEYWORDS: Amazon turtles, Blood, leukocytes, *Podocnemis expansa*, Thrombocytes.

Limitações metodológicas de contagens de leucócitos e trombócitos totais em répteis (tartaruga da Amazônia, *Podocnemis expansa*): uma análise e discussão

RESUMO

O objetivo deste estudo foi comparar três diferentes métodos para contar leucócitos totais [LT] (método de Natt & Herrick, de estimação em 1000 e 2000 eritrócitos) e três métodos para contar trombócitos totais [TT] (método de Wojtaszek, de estimação em 1000 e 2000 eritrócitos) em uma espécie de tartaruga de água doce da América do Sul, *Podocnemis expansa*, Schweigger 1812 (Reptilia, Pelomedusidae). As contagens diretas de LT usando o método de Natt & Herrick mostraram limitações que são aqui discutidas. As contagens de LT e TT usando estimativa em 1000 eritrócitos na extensão sanguínea não são recomendadas para tartaruga-da-Amazônia nem para outras espécies de répteis, pois houve ampla variação nestas contagens. Os métodos para determinar LT e TT baseados em 2000 eritrócitos nas extensões sanguíneas foram mais aceitáveis porque eles permitem uma diferenciação entre leucócitos e trombócitos, além disso, teve uma variação menor. Os métodos aqui investigados para tartaruga-da-Amazônia, os quais são amplamente usados em outras espécies de répteis, proveram evidências de que o método mais aceitável não é o que usa corantes diluentes e um hemocitômetro.

PALAVRAS-CHAVE: Tartaruga-da-Amazônia, Sangue, Leucócitos, *Podocnemis expansa*, Trombócitos.

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INTRODUCTION

Historically there has been little or no controversy for quantification of blood cells in mammalian species by automated methods. The development of lysis techniques to facilitate counting mammalian white blood cells (WBC) by removing the non-nucleated erythrocytes inaugurated the distinction between mammalian and lower vertebrate hematology because platelets are present in mammalian cell diluents.

The establishment of baselines values for total WBC and total thrombocytes (TT) of reptilian species has been hindered for several reasons. Heparin is the anticoagulant recommended for blood collection in chelonians (Muro *et al.*, 1988; Knotková *et al.*, 2005) because the ethylene diamine tetra acetic acid (EDTA) causes hemolysis. However, in others reptilian species, it has been reported that heparin lead to aggregation of leukocytes (Cuadro *et al.*, 2003) and thrombocytes (Salakij *et al.*, 2002). Aggregates accumulate on the edges of blood smears and then WBC and TT cannot be counted, increasing the error. Therefore, the selection of the best anticoagulant used for hematologic determinations needs to be determined for the reptilian species being evaluated.

It is not possible to separate the nucleated cells and obtain an automated count. Thus, several differential staining diluents developed for direct WBC counts in birds and fish using a hemocytometer have been also used for different reptilian species. Manual analysis methods for counting WBC and TT in low vertebrates, including reptiles, have gained wider acceptance than automated methods because the latter are not easily applied. Estimated WBC and TT counts based on an actual erythrocytes count method, and the ratio of leukocytes and thrombocytes to erythrocytes taken from blood smears can be performed (Wojtaszek, 1991; Millan *et al.*, 1997; Work *et al.*, 1998; Keller *et al.*, 2004), however, it depends heavily on the methods used to obtain the red blood cell (RBC) counts. Consequently, most discussions on reptile hematological methodology are in relation to the leukocytes and thrombocytes number. The introduction of a new species to a methodology that has been previously validated for other species has to be part of the quality control necessary for any veterinary clinical laboratory (Walton, 2001). Therefore, this study was designed not only to compare different methods for counting WBC and TT in Amazon turtles *Podocnemis expansa*, but also to evaluate the viability and limitations of various methods used for this purpose in reptiles. Hence, this investigation is of interest, first, because it presents blood cell values from an important Amazon chelonian, which has not yet been studied, and second, because it highlights factors that complicate the interpretation of reptilian clinical data.

MATERIAL AND METHODS

Healthy Amazon turtles *Podocnemis expansa* (weighing from 0.50 to 12.75 Kg) were collected from a culture farm from Manaus (Amazonas State, Brazil). Blood samples were collected from 27 turtles from the caudal vessel into syringes coated with 0.01 mg/ml sodium heparin (2.500 UI.ml^{-1}). Total red blood cell (RBC) and white blood cell (WBC) counts (/µl) were determined by the Natt & Herrick (1952) method (NH) using a Newbauer hemocytometer. In order to prevent potential the artifact from anticoagulant, in terms of both clumping and staining quality besides samples degradation, heparin (5000 UI.ml^{-1}) was diluted in 0.65% sodium chloride to ratio 1:1. In addition, blood smears were made immediately after obtaining the blood samples and they were actively air-dried, and then stained with a combination of May Grünwald-Giemsa-Wrigth (Tavares-Dias & Moraes, 2003), a Romanowsky-type stain. These blood smears were examined at 1000× magnification for the three methods for estimating TT and the two methods for WBC counts. In the first TT method, proposed by Wojtaszek (1991), the number of thrombocytes was then recalculated per unit volume as follows: TT/µl= (thrombocyte count to 200 WBC per blood smear) × (WBC count per µl of blood)/(200). Two others methods were the same for both TT and WBC. Results were determined by counting the number of cells from blood smear, either WBC or TT, based on 1000 RBC (estimation method 1) or on 2000 RBC (estimation method 2) and then recalculated per unit volume as follows: TT or WBC/µl= cell count (either thrombocytes or leukocytes) to 1000 or 2000 RBC per blood smear × (RBC count per µl of blood)/(either 1000 or 2000 RBC) (Tavares-Dias & Moraes, 2006).

RESULTS

Estimation of the WBC count performed in 2,000 erythrocytes of blood smears in *P. expansa* had results which were not similar to those obtained using the NH method with a hemocytometer or 1,000 RBC of blood smears. The estimation methods for determining WBC per 1,000 and 2,000 RBC from blood smears were correlated (Table 1). The diluents of the NH method does not allow for adequate differentiation between leukocytes and thrombocytes. The TT count estimates of 1,000 or 2,000 RBC from blood smears showed similar results to that of the method described by Wojtaszek (1991). Of these, only count estimates between 1,000 and 2,000 RBC were statistically correlated (Table 2).

DISCUSSION

In lower vertebrates, the presence of nucleated erythrocytes and thrombocytes precludes the use of the automated methods

Table 1 - Mean values ± standard deviation (SD), range, coefficient of variation (CV) and linear regression for WBC counts (μL) in *P. expansa* obtained by different methods. Different letters indicate significant differences between methods by Dunn's test (P<0.001).

Methods	N	Mean ± SD	Range	CV (%)	Linear Regression Equation	r	P
Natt and Herrick (1952)	27	9,407.0 ± 3,697.0[a]	2,000.0–15,000.0	39.3	Estimation 1=2053.896+(131.377*NH)	0.239	0.229
Estimation 1	27	3,290.0 ± 2,030.0[b]	480.0–9,900.0	61.7	Estimation 2=3894.618+(231.458*NH)	0.364	0.062
Estimation 2	27	6,072.0 ± 2,349.0[c]	2,480.0–10,450.0	38.7	Estimation 1=-316.425+(0.594*Estimation 2)	0.687	0.001

used for counting WBC. Therefore, WBC counts may be performed either by indirect or direct method (Campbell, 2004). In *P. expansa*, the NH method presented limitations for determining the WBC, exhibiting an inefficient staining of these cells in the hemocytometer. This may also be the case for other reptilian species where not only do the WBC stain dark blue but also immature erythrocytes and other structures. In contrast, Salakij *et al.* (2002) reported that the leukocytes nuclei of king cobra *Ophiophagus hannah*, stained dark blue in the NH diluents whereas thrombocytes nuclei were unstained or very pale blue in appearance. Therefore, these results indicate the importance of method validation for different species, because as a result of the interspecies structural differences in any given analysis, a methodology that is adequate for one species may be inappropriate for another (Walton, 2001).

In loggerhead sea turtle *Caretta carretta* (Keller *et al.*, 2004), no difference was found between the NH method, a direct method, and the indirect method by estimation from blood smears for counting WBC. In contrast, in *P. expansa*, the NH method differs in relation to the estimation method for counting WBC from 2,000 RBC of blood smears. Hence, the results of turtle WBC as well as other reptiles, when using the NH method should be used with caution in future studies. Nevertheless, estimation methods are subject to variability from factors such as discrimination between leukocytes and thrombocytes, blood smear quality, upon uniform distribution of cells, and absence of cell lysis in the sample.

In healthy reptiles, the coefficient of variation (CV) derived from WBC counts, which have been performed by several methods, may vary from 10.95 to 119.4% (Wood *et al.*, 1984; Mateo *et al.*, 1984; Pajés *et al.*, 1992; Troiano & Silva, 1998; Work *et al.*, 1998; Troiano *et al.*, 2000; Salakij *et al.*, 2002; Pejrilová *et al.*, 2002; Raphael, 2003; Schumacher, 2003; Knotková *et al.*, 2005). In *Chamaeleo chamaeleon*, it has been reported that NH method showed a low repeatability and a high CV due to a low concentration of leukocytes in the blood samples, which resulted in higher count errors. Cell aggregation and high variation in cell counts produced by the use of heparin were also been reported (Cuadro *et al.*, 2003). However, in *P. expansa* we do not observe leukocyte aggregation using diluted heparin in sodium chloride solution. In addition, the CV of WBC from 2,000 RBC of blood smear was similar to the NH method results, and both methods had a CV similar to that obtained from other reptiles using estimation methods for counting the WBC. In contrast, both these methods presented higher variations, compared to mammals, for which the CV values are well established. Nevertheless, the estimation methods for determining WBC and TT per 1,000 RBC from blood smears can not recommended neither for Amazon turtles nor for other reptiles, because a higher variation in counting was found.

Reptile thrombocytes, including chelonian species, play a role in hemostasis (Campbell, 2004; Pellizzon & Lunardi, 2000) and may also have a great potential for phagocytosis (Pellizzon & Lunardi, 2000). Despite the important function

Table 2 - Mean values ± standard deviation (SD), range, coefficient of variation (CV) and linear regression for TT counts (μL) in *P. expansa* obtained by different methods. Mean values are not different by Dunn's test (P<0.05)

Methods	N	Mean ± SD	Range	CV (%)	Linear Regression Equation	r	P
Wojtaszek (1991)	27	4938.0 ± 2237.0	518.0–9777.0	66.5	Estimation 1=2794.706+(0.295*Wojtaszek)	0.234	0.241
Estimation 1	27	4251.0± 2826.0	1120.0–11590.0	66.5	Estimation 2=1993.319+(0.383*Wojtaszek)	0.502	0.008
Estimation 2	27	3882.0 ± 1706.0	2080.0–9900.0	43.9	Estimation 1=3967.028+(0.0733*Estimation 2)	0.044	0.827

of the thrombocytes, studies reporting TT counts have been few and sporadic in reptiles (Wojtaszek, 1991; Work *et al.*, 1998), which in part can be attributed to the lack of an direct adequate method for this purpose (Wojtaszek, 1991). Therefore, a TT count from blood smears can be performed (Campbell, 2004). In *P. expansa*, although the TT counting methods used provided similar mean values, they showed limitations when compared to clinical purposes, since the acceptance among them do not meet the criteria established for test methods and comparison (Walton, 2001). The Wojtaszek (1991) method estimation is based on 200 WBC per blood smear and is less time consuming. However, it has limitations compared with the other two methods of estimation evaluated. The Wojtaszek method has not been used in reptiles because it requires WBC count which may also be performed by estimation rather than by direct counting and can consequently cause a high variation in the final TT result, as observed in the Amazon turtle. For this species, the estimation made with 1,000 RBC for counting TT showed high variation, contrasting with estimations made with 2,000 RBC which revealed a lower and more suitable CV, but still higher to that obtained from many reptiles (Taylor & Jacobson, 1982; Mateo *et al.*, 1984; Wojtaszek, 1991; Troiano & Silva, 1998; Troiano *et al.*, 1999; Troiano *et al.*, 2000; Martinez-Silvestre *et al.*, 2004). Moreover, estimation of TT using blood smears is also subject to variability due to some factors which were already highlighted above. In *P. expansa*, 2.500 IU/ml sodium heparin had an efficient anticoagulant effect and only occasionally very moderate thrombocyte aggregation in some blood smears was found. In contrast, in Hermann's tortoises *Testudo hermananni* when lithium heparin was used for collecting blood, no thrombocyte aggregation was reported (Muro *et al.*, 1998). On the other hand, in blood smears *C. caretta* performed with blood collected without anticoagulant has been also reported tendency to thrombocytes aggregation (Casal & Orós, 2007).

Estimation of total WBC and TT has been widely used for reptilian species yet it does not provide absolute counts. However, WBC and TT determination from blood smears are extremely dependent on the ratio of leukocytes or thrombocytes to erythrocytes. In contrast, the hemocytometer methods that use specialized diluents have been accurate in WBC counts for some species, whereas for others its precision and selectivity does not allow adequate differentiation between the leukocytes and thrombocytes. Therefore, there is an urgent need to develop accurate methods with higher precision, preferentially, automated methods such as those that are already standardized for mammalian species. Only by establishing reliable standard methods, can we conduct comparative studies by species rather than rely upon the large discrepancy found in literature. This information would be useful for health assessment of captive and wild reptiles.

ACKNOWLEDGMENTS

The authors gratefully acknowledge research grants provided by FAPEAM (Fundação de Amparo à Pesquisa do Estado do Amazonas, #2203/05 and #2204/05), and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, #35.0117/20005-5).

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Recebido em 13/07/2007
Aceito em 21/12/2007