

TOTAL LEUKOCYTE COUNTS IN FISHES BY DIRECT OR INDIRECT METHODS?

[Contagem de leucócitos totais em peixes por métodos direto ou indireto?]

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ABSTRACT

Several methods have been recommended or adapted to count total leukocytes in fishes, but the efficiency of diluents used in these methods are questioned. In this trial, total leukocyte count of *Piaractus mesopotamicus* Holmberg, 1887 (Characidae) was evaluated using three different methods, one direct and two indirect. The mean values of leukocyte counts by indirect methods were not statistically different ($P>0.05$). However, these values were higher than those resulting from the direct method (recommended by NATT and HERRICK, 1952). The low values obtained by the latter may be due to inefficient staining of leukocytes in the hemocytometer. Further studies are required for resolving this question. Therefore, fish hematology researchers must direct their efforts to find a methodology that allows accurate total leukocyte counting.

Key words: leukocytes; fishes; *Piaractus mesopotamicus*; blood; hematology

RESUMO

Diversos métodos foram preconizados ou adaptados para contagem de leucócitos totais em peixes, porém a eficiência dos diluentes usados nesses métodos tem sido questionada. Neste ensaio foi avaliada a contagem de leucócitos totais em *Piaractus mesopotamicus* Holmberg, 1887 (Characidae), utilizando três diferentes métodos, um direto e dois indiretos. Os valores médios da contagem de leucócitos pelos métodos indiretos não diferem entre si estatisticamente ($P>0,05$), porém ambos mostraram valores médios superiores ao do método direto (preconizado por NATT e HERRICK, 1952). Os baixos valores da contagem de leucócitos obtidos com esse método direto podem ser devidos à ineficiência de coloração dos leucócitos no hemocitômetro. Assim, outros estudos são necessários para resolver esta questão, sendo urgente que os pesquisadores em hematologia de peixes direcionem sua atenção para tal problema, no sentido de encontrar uma metodologia que permita a quantificação precisa dos leucócitos totais.

Palavras-chave: leucócitos; peixes; *Piaractus mesopotamicus*; sangue; hematologia

Introduction

Some hematological methods routinely used for determining blood values in mammals have also been successfully used in fishes. An exception is total leukocyte count. The first method for counting total leukocytes and nucleated erythrocytes was proposed by Warthin in 1907, for birds (NATT and HERRICK, 1952; KEKIC and IVANC, 1982). Later, different methods (Yokoyama, 1947, *apud* NATT and HERRICK, 1952; BLAXHALL and DAISLEY, 1973; CHRISTENSEN; FIANDT; POESCHL, 1978; PITOMBEIRA and MARTINS, 1966; KEKIC and IVANC, 1982; BERRA *et al.*, 1993; INOUE *et al.*, 2002) were recommended for total leukocyte counting in fishes, with the use of hemocytometer. However, the method of SHAW (1930), developed for birds blood, is widely used

by researchers (FARGHALY; EZZAT; SHABANA, 1973; EZZAT; SHABANA; FARGHALY, 1974; MURRAY and BURTON, 1979; MAHAJAN and DHEER, 1979; SOPINSKA, 1983; MAHAJAN and DHEER, 1983; RAMBHASKAR and SRINIVASA-RAO, 1987; UEDA *et al.*, 1997; RAHKONEN and PASTERNAK, 1998).

Another successful method used for leukocyte counting in birds is that proposed by NATT and HERRICK (1952), and thus has been tested in different teleosts (YILDIZ, 1998; TAVARES-DIAS *et al.*, 1999a,b,c; MARTINS, 2000; MARTINS *et al.*, 2001; TAVARES-DIAS *et al.*, 2001), which also have nucleated erythrocytes and thrombocytes. BLAXHALL and DAISLEY (1973) adapted a direct method for total leukocyte counting in fishes, which was used by researchers, such as GAVIRIA and PÉREZ, 1979; LEA MASTER *et al.*, 1990; LAMAS *et al.*, 1994; THOMAS *et al.*, 1999.

Indirect methods for the quantification of total leukocytes use to count erythrocytes in hemocytometer and the number of leukocytes and erythrocytes in blood extensions previously stained with routine panoptics stains. These methods have been commonly used for the determination of total cell count in several teleost species (McKNIGHT, 1966; PITOMBEIRA; MARTINS; FURTADO, 1968; MARTINS and PITOMBEIRA, 1968; PITOMBEIRA; GOMES; MARTINS, 1969; CHLEBECK and PHILLIPS, 1969; PITOMBEIRA and MARTINS, 1970; HINES and SPIRA, 1973; BREAZILE *et al.*, 1982; ALVAREZ-PELLITERO and PINTÓ, 1987; RANZANI-PAIVA *et al.*, 1987).

Direct methods for the quantification of total leukocytes in fishes usually use diluents with substances that stain these cells, allowing their counting in the hemocytometer. PITOMBEIRA and MARTINS (1966) did not find differences between direct and indirect method. Despite the recommendations of methods using special diluents for leukocyte countings in fishes, the indirect method is considered more accurate (McKNIGHT, 1966; HINES and YASHOUV, 1970). Therefore, the present study

aimed at comparing three different methods, one direct and two indirect, to total leukocytes counts in *Piaractus mesopotamicus*.

Material and Methods

Forty-eight specimens of *Piaractus mesopotamicus* (weighing between 102.80 and 324.23 g), from the same hatching, were collected in Aquaculture Center of UNESP (CAUNESP), Jaboticabal, São Paulo, Brazil, and submitted to hematological test.

A blood aliquot (0.5 mL) was collected from the caudal vessel with the aid of syringes containing EDTA (10%). Blood was used for total erythrocyte count (RBC) in Neubauer chamber, leukocyte count (WBC) using the diluent of NATT and HERRICK (1952) (3.88 g NaCl; 2.5 g Na₂SO₄; 2.91 g Na₂HPO₄·12H₂O; 0.25 g KH₂PO₄; 7.5 mL formalin, 0.10 g methyl violet 2B), Neubauer chamber and confection of blood extensions dyed with May Grünwald-Giemsa-Wright (TAVARES-DIAS, 2002). Blood extensions were used to total leukocytes counts by two different indirect methods:

1. Method proposed by PITOMBEIRA and MARTINS (1966)

$$\text{Leukocytes/mL blood} = \frac{\text{number of leukocytes in extension} \times \text{erythrocyte count in Neubauer chamber}}{5000 \text{ erythrocytes in blood extension}}$$

2. Indirect method (which is used by our laboratory). In this method, the number of erythrocytes and that of leukocytes are registered counted in 10 homogenous fields of each blood extension. These results and erythrocyte counting made in Neubauer chamber for each fish are used in the following calculation:

$$\text{Leukocytes/mL blood} = \frac{\text{number of leukocytes in extension} \times \text{erythrocyte count in Neubauer chamber}}{\text{number of erythrocytes in blood extension}}$$

The same person did the count total leukocytes by two different indirect methods.

Statistical analysis used a completely randomized experimental design with three treatments. Mean values were compared by the Tukey test at 5% probability after data were transformed in Neperian logarithms, as the distribution of data was not normal. The software Statistical Analysis System/SAS was used.

Results and Discussion

In this trial, mean values obtained in total leukocyte counts of *P. mesopotamicus* by both indirect methods were not statistically different ($P > 0.05$). However, both methods had lower coefficients of variation and higher mean values as compared to the method developed by NATT and HERRICK (1952). Values not submitted to transformation are shown in table 1.

Table 1. Mean values $\pm$ standard deviation (SD), amplitude of variation (AX) and coefficient of variation (CV) of total leukocyte counts (n°/mL) in *Piaractus mesopotamicus* (N=48), obtained by three different methods. Mean values followed by the same letter are not statistically different by the test of Tukey ($P > 0.05$)

Method	Mean value $\pm$ SD	AX	CV(%)
Indirect	18730.8 $\pm$ 9721.1 ^A	2019.0–47473.0	7.5
PITOMBEIRA and MARTINS (1966)	17827.8 $\pm$ 8837.7 ^A	5940.0–44820.0	7.1
NATT and HERRICK (1952)	3636.7 $\pm$ 3004.7 ^B	400.0–13200.0	11.9

The low leukocyte values obtained in the present study using the diluent of NATT and HERRICK seem to result from inefficient staining of leukocytes in Neubauer chamber. In addition, this diluent does not allow the differentiation between leukocytes and thrombocytes during countings in Neubauer chamber. KEKIC and IVANC (1982) also reported staining deficiency of this diluent in leukocyte counting in fishes. The stain used in NATT and HERRICK's diluent stains leukocytes in dark blue, allowing their counting in Neubauer chamber. Although they are intensely stained, considerable experience is necessary to differentiate them from leukocyte fragments, immature erythrocytes, and other structures, which are also intensely stained (DEIN *et al.*, 1994). However, this diluent is recommended by STOSKOPF (1993) to quantify total leukocytes in teleost fishes.

Automatic leukocyte counting in mammals has a coefficient of variation (CV) of 3.0-4.0% (DACIE and LEWIS, 1991), indicating that data dispersion

in relation to the mean value is small, that is, dispersion is relatively low. DEIN *et al.* (1994), comparing total leukocyte counts in four bird species by two different methods (NATT and HERRICK's diluent and Unopette), found a CV of 14.2%, with a range of 5.5% to 17.2%. Those authors mention that literature shows CV values for birds ranging from 11.0% to 34.0%. CVs derived from total leukocyte counts in teleost fishes may vary between 8.0% and 49.8% (Table 2). In the present study, CV ranged from 7.1% to 11.9%, which is an acceptable level. Interestingly, in the sea fish *Scomberomorus maculatus* and *Opisthonema oglinum*, CV of leukocyte counts was 80.1% and 89.7%, respectively (PITOMBEIRA; MARTINS; FURTADO, 1968). This shows that the dispersion of data in relation to the mean is very large. However, in the freshwater fish *Rhamdia hilarii*, CV of total leukocyte counts is 30.8%, with variation between 16.0 and 25.0% (KAVAMOTO; RANZANI-PAIVA; TOKUMARU, 1983).

Table 2. Coefficient of variation (CV= standard deviation / mean x 100) of total leukocyte counts (n°/mL), in several teleosts, calculated from literature studies. * CV provided by the author

Species	CV(%)	N	Reference
<i>Ictiobus cyprinellus</i>	43.8*	10	CHLEBECK and PHILLIPS (1969)
<i>Ictiobus bubalus</i>	36.3*	10	CHLEBECK and PHILLIPS (1969)
<i>Cyprinus carpio</i>	12.4-29.6	20	SOPINSKA (1983)
<i>Ictalurus punctatus</i>	49.8	33	BREAZILE <i>et al.</i> (1982)
<i>Mugil curema</i>	19.5	68	GAVIRIA and PÉREZ (1979)
<i>Labeo rohita</i>	21.2	29	SIDDIQUI and NASEEM (1979)
<i>Rhamdia hilarii</i>	30.8*	18	KAVAMOTO; RANZANI-PAIVA; TOKUMARU (1983)
<i>Gila elegans</i>	27.9	20	BERRY (1984)
<i>Gila cypha</i>	25.3	30	BERRY (1984)
<i>Xyrauchen texanus</i>	32.1	56	BERRY (1984)
<i>Ptychocheilus lucius</i>	37.2	29	BERRY (1984)
<i>Oreochromis niloticus</i>	8.0	10	UEDA <i>et al.</i> (1997)
<i>Megalopops cyprinoides</i>	8.2	70	RAMBHASKAR and SRINIVASA-RAO (1987)
<i>Mugil cephalus</i>	19.6	35	RAMBHASKAR and SRINIVASA-RAO (1987)
<i>Rastrelliger kanagurta</i>	26.5	17	RAMBHASKAR and SRINIVASA-RAO (1987)
<i>Colossoma macropomum</i>	48.3	30	TAVARES-DIAS <i>et al.</i> (1999a)
<i>Brycon cephalus</i>	48.5	20	TAVARES-DIAS <i>et al.</i> (1999c)
<i>Pimephales promelas</i>	39.6	38	THOMAS <i>et al.</i> (1999)
<i>Piaractus mesopotamicus</i>	17.1	10	TAVARES-DIAS <i>et al.</i> (1999b)
<i>Piaractus mesopotamicus</i>	31.9	10	MARTINS <i>et al.</i> (2001)

Due to the factor anteriorly mentioned, the two indirect methods used in this study can be considered more accurate than the method of NATT and HERRICK (1952). Other indirect methods are also considered efficient in leukocyte counting of *Prosopium williamsoni* (McKNIGHT, 1966) and of *Cyprinus carpio* (HINES and YASHOUV, 1970). McKNIGHT (1966), comparing leukocyte counts by an indirect method (number of leukocytes in the extension x erythrocytes quantified in the hemocytometer/7000 erythrocytes in the extension) with those by a direct method employing Yokoyama diluent, concluded that the first method shows more accurate results. Lucas and Jamroz (1961), *apud* McKNIGHT (1966), reported that the both the direct and the indirect method of leukocyte counting result in errors, but they recommend the latter one. However, a similar study did not show differences between the indirect and the direct method in *S. maculatus* (PITOMBEIRA and MARTINS, 1966). The methods proposed by McKNIGHT (1966) and PITOMBEIRA and MARTINS (1966) recommend a higher quantification of erythrocytes in blood extensions. These methods are similar, but the first recommend a quantification of the 7000 erythrocytes in blood extensions and the second, 5000 erythrocytes. Therefore, the method employed in our laboratory consumes less time and labor.

Indirect methods are used to count the number of leukocytes and of erythrocytes in blood extensions previously stained with panoptics, and thus, are extremely dependent on the uniformity of these cells in the extensions (STOSKOPF, 1993). NATT and HERRICK (1952) question the precision of these methods in the quantification of leukocytes, as they assume same distribution of blood cell in the extension. The authors argue that this may not be true, as polymorphonuclear cells tend to concentrate along the borders of the extension. Therefore, it is essential to make all blood extension in the same way and by a single technician in order to avoid distortions in the results.

The problem of leukocyte counting in fishes has still to be solved, despite the recommendation of methods using special diluents, which do not destroy thrombocytes and erythrocytes – their cytoplasm is dissolved, but the nucleus remains intact. The modified method of BLAXHALL and DAISLEY (1973), based on Dacie's diluent (formaldehyde, tri-sodium citrate, distilled water), with the addition of bright crescyl blue, is considered relatively effective to count leukocytes in fishes, as the factor of dilution is high, resulting in a high coefficient of variation. Moreover, this stain is difficult to remove from pipettes used for

dilution (BERRA *et al.*, 1993).

The method of SHAW (1930) (neutral red, NaCl, crystal violet, sodium citrate, formaldehyde, distilled water) is also recommended for leukocyte counting in fishes due to its efficiency (HESSER, 1960). MAHAJAN and DHEER (1979; 1983) report that this diluent allows the simultaneous counting of erythrocytes and leukocytes. However, other authors report problems in reading (BERRA *et al.*, 1993) and the instability of the stains used in this method (BLAXHALL, 1972; KEKIC and IVANC, 1982). Thus, this stain solution must be prepared daily. Also, the concentration of fixer of the stain of the diluent of Shaw is inadequate (KEKIC and IVANC, 1982).

A diluent consisting of Giemsa, NaCl, $\text{Na}_2\text{HPO}_4 \times 12\text{H}_2\text{O}$, ethylene-di-amine tetra-acetic acid (EDTA) and formaldehyde was recommended to the simultaneous counting of erythrocytes, leukocytes, and total thrombocytes in fishes (KEKIC and IVANC, 1982). However, when we tested the diluent of KEKIC and IVANC in our laboratory, it did not allow the differentiation of these three cell types. This was probably due to problems in the preparation of the diluent, as the described methodology was not fully repeatable. Another method for counting leukocytes in fishes was adapted by adding the hematological stain of Wright to the diluent of Klontz (NaCl, KCl, formaldehyde, dextrose, NaHCO_3 and distilled water). The latter is derived from the method originally described by Yokoyama in 1947 (CHRISTENSEN; FIANDT; POESCHL, 1978). However, these methods do not produce satisfactory results, as they stain only monocytes and polymorphonuclear cells. A new method for total leukocyte counting in teleost fishes was recently adapted from a diluent recommended for birds (BERRA *et al.*, 1993). The disadvantage of this method is that it does not allow leukocytes to be counted in the same day of the dilution, as it requires 24 hours of previous storage at 4°C. When tested in our lab, the diluent of this method also did not show effective staining.

A diluent consisting of 3,3-dihexyloxacarboxyanine $\text{DiOC}_6(3)$ and Hanks's saline solution was recently tested for the differentiation and counting of blood cells in fishes, birds, and reptiles. The results showed that, in *C. carpio*, *Carassius auratus*, *Oncorhynchus mykiss* and *Misgurnus anguillicaudatus*, the stain $\text{DiOC}_6(3)$ was efficient to differentiate leukocytes from erythrocytes. However, it also stains thrombocytes. This stain can be useful when employed in leukocyte counts that use monoclonal antibodies against thrombocytes

(INOUE *et al.*, 2002). Thus, these results may originate new studies that will perhaps solve the problems of total leukocyte counts in fishes.

Literature shows that several researchers use direct methods for the quantification of total leukocyte counting in fishes (CHLEBECK and PHILLIPS, 1969; HINES and YASHOUV, 1970; BLAXHALL and DAISLEY, 1973; FARGHALY; EZZAT; SHABANA, 1973; EZZAT; SHABANA; FARGHALY, 1974; CHRISTENSEN; FIANDT; POESCHL, 1978; MAHAJAN and DHEER, 1979; MURRAY and BURTON, 1979; MAHAJAN and DHEER, 1983; KAVAMOTO; RANZANI-PAIVA; TOKUMARU, 1983; SIDDIQUI and NASEEM, 1979; SOPINSKA, 1983; RAMBHASKAR and SRINIVASA-RAO, 1987; LEA MASTER *et al.*, 1990; KUROVSKAYA and OSADCHAYA, 1993; LAMAS *et al.*, 1994; HOUSTON; DOBRIC; KAHURANANGA, 1996; UEDA *et al.*, 1997; RAHKONEN and PASTERNAK, 1998; YILDIZ, 1998; FORERO, 1999; TAVARES-DIAS *et al.*, 1999a,b,c; MARTINS, 2000; MARTINS *et al.*, 2001), whereas others use indirect methods in their studies (McKNIGHT, 1966; PITOMBEIRA; MARTINS; FURTADO, 1968; MARTINS and PITOMBEIRA, 1968; PITOMBEIRA; GOMES; MARTINS, 1969; CHLEBECK and PHILLIPS, 1969; PITOMBEIRA and MARTINS, 1970; HINES and SPIRA, 1973; BREAZILE *et al.*, 1982; ALVAREZ-PELLITERO and PINTÓ, 1987; RANZANI-PAIVA *et al.*, 1987).

In summary, all factors discussed here show the urgent need to develop a method that allows higher precision in leukocyte counting in fishes, preferably an automated method, as those already used for other vertebrates with non-nuclear erythrocytes.

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