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Research Article
Cell Axis Ratio a Standardized Approach for Normal Erythrocyte Shape Determination in Fishes

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Abstract

Background and Objective: Erythrocyte shape determination has largely been gone through visual observation only. This study describes for the very first time a standardized method for normal erythrocyte shape determination in fishes using the values of the cell axis ratio. **Materials and Methods:** Blood smear was made from two important freshwater species namely *Pangasianodon hypophthalmus* and *Clarias gariepinus*. Erythrocyte measurements of the cell and nuclear axis were done under the microscope, while the cell axis ratio was calculated (as minor axis/major axis). Visual assessment was done to categorize the erythrocyte into two (rounded and oval) or three (strongly rounded, slightly oval and strongly oval) groups. This was then matched with its corresponding cell axis ratio to formulate two standardized decision rules for shape determination. Descriptive statistics of the measured and calculated parameters were done using Minitab 14. **Results:** Decision rule Type A: Rounded erythrocyte have cell axis ratio between 1.0 and 0.75, while the oval cell is below 0.75. Decision rule Type B: Strongly rounded erythrocyte have cell axis ratio between 1.0 and 0.90, slightly oval cell is between 0.89 and 0.75 and strongly oval cell below 0.75. **Conclusion:** This novel approach to shape determination of normal fish erythrocyte proposed in this study have been shown to be easier, simpler and accurate than visual observation alone, hence, preventing false generalization.

Key words: African catfish, Asian catfish, cell axis ratio, erythrocyte shape, visual assessment

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Erythrocyte characterization has been used as a fast and cost-effective biomarker for environmental mutagenesis[1-3] and diploid-triploid discriminating in fish species[4-8]. Erythrocytes of different fish species differ in shape and size and are easily affected by environmental imbalances. Alteration in the shape and sizes of normal erythrocyte are effective indicators of cytotoxicity[9-11]. Cell shrinkage, membrane blebbing, chromatin condensation, cell swelling, tear drop-like cells, sickle cells to mention but a few are examples of shape alteration which are consequences of environmental imbalances[9,10,12-14]. The unique shape and small percentages occurrence of the abnormal erythrocyte cells make it easy for classification and quantification.

Despite the fact that some fish erythrocyte comprises of both rounded and oval shapes in different proportions, shape proportion is seldom reported in many previous studies. More so, studies have shown that erythrocyte shape differs when taken from different parts of the fish and at different stages of development[7]. The study by Nilsson *et al*.[15] had demonstrated the fact that erythrocyte of *Oncorhynchus mykiss* and roach *Rutilus rutilus* were elliptical in the arterioles and arteries but rounded in the gills pillar cells. Hence, the determination of shape proportion could be pivotal for accurate erythrocyte characterization. Many previous studies as described above rely on visual observation alone to describe the shape of erythrocyte, hence leading to high level of false generalization due to human error. Till date, there is no standardized method for shape determination. Hence, for the purpose of simplicity and accuracy, it was attempted to design for the very first time a standardized protocol for discriminate rounded and oval shaped erythrocyte in fish with particular references to Asian catfish *Pangasianodon hypophthalmus* and African catfish *Clarias gariepinus*. The decision rules drawn for this study were proposed from the result of visual assessment and the equivalent values of cell axis ratio.

MATERIALS AND METHODS

Ten fingerlings of *P. hypophthalmus* and *C. gariepinus* were gotten from the School of Fisheries and Aquaculture Sciences hatchery of the Universiti Malaysia Terengganu, Malaysia in January, 2017. Blood was collected from the caudal peduncle of the fish using an 18 gauge needle fitted with a heparinized syringe. Dry blood smear was then prepared using the method previously specified by Felip *et al*.[16], Felip *et al*.[17] and Jalil *et al*.[7]. The slide was observed under the microscope (Nikon eclipse 80i) at 100× magnification. Five hundred

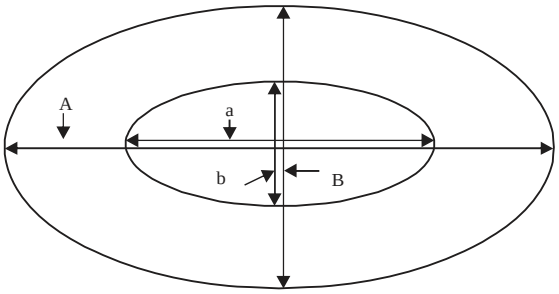

Fig. 1: Sketch of erythrocyte with measurement for A (cell major axis), B (cell minor axis), a (nucleus major axis) and b (nucleus minor axis) (Sketch adapted from Normala *et al*.[7])

erythrocytes were measured in total for each group characterized using the slide sectioning method described by Jalil *et al*.[7]. The parameters measured were cell major axis, cell minor axis, nucleus major axis and nucleus minor axis (Fig. 1). From these parameters, the cell area, nucleus area, cell volume, nucleus volume and cell axis ratio were calculated using the formulae below[7]:

$$\text{Area of erythrocyte} = \pi \times A \times B$$

$$\text{Volume of erythrocyte} = 4/3 \times \pi \times (A/2) \times (B/2)^2$$

$$\text{Cell axis ratio} = \frac{\text{Cell minor axis}}{\text{Cell major axis}}$$

To standardize shape determination in the erythrocyte of fish, ten visual assessors were invited to observe and score pictures of fifty erythrocytes (numbered serially for ease of tracking) (Fig. 2). Assessors were initially asked to score erythrocytes as rounded or oval. The process was repeated however with the addition of a third class of erythrocyte namely "slightly oval" (and the former classes changed to strongly rounded or oval). The cell axis ratio of all scored erythrocyte was then determined and aligned with assessor's scores so that a standardized decision rule could be formulated for shape determination. Using these rules, the proportion of the different shapes of erythrocyte (in percentages) observed in the two species of reference in this study was determined and results presented in a bar chart using the Microsoft excel software.

Statistical analysis: Descriptive statistics of the measured and calculated parameters were done using Minitab 14 (Minitab inc. State college, Pennsylvania, USA) computer software. Student's t-test was done to determine if there

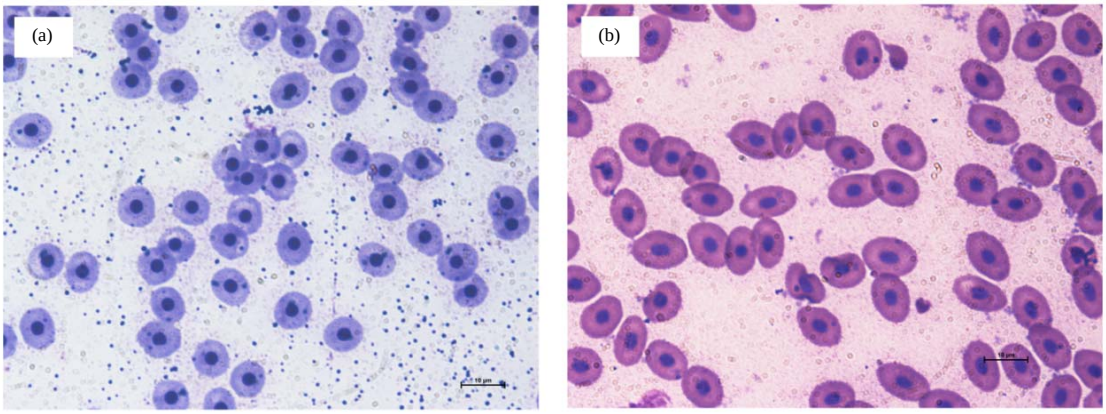

Fig. 2(a-b): Erythrocytes of (a) *Clarias gariepinus* and (b) *Pangasianodon hypophthalmus*

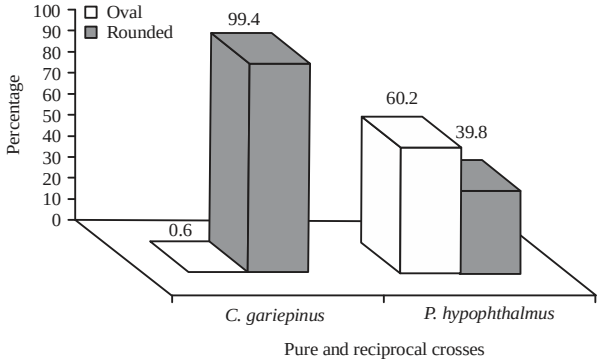

Fig. 3: Proportion of erythrocyte shape in *Clarias gariepinus* and *Pangasianodon hypophthalmus* using "Type A" decision rule

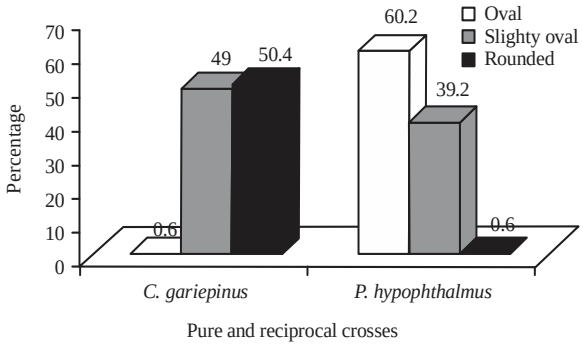

Fig. 4: Proportion of erythrocyte shape in *Clarias gariepinus* and *Pangasianodon hypophthalmus* using "Type B" decision rule

are significant variations ($p \leq 0.05$) in erythrocyte parameter of African and Asian catfish.

RESULTS

Based on the scores of the assessors for each erythrocyte and their equivalent cell axis ratio, two standardized decision rules are designed for this study namely "Type A" and "Type B". For "Type A" decision rule, cell axis ratio greater or equal to 0.75 was regarded as rounded, while those lower than 0.75 were regarded as oval. For "Type B" decision rule, cell axis ratio greater or equal to 0.90 were regarded as strongly rounded, cell axis ratio between 0.89 and 0.75 was regarded as slightly oval, while those below 0.75 were regarded as strongly oval.

Using the two decision rule, the proportion of the shape of 500 erythrocytes each for *P. hypophthalmus* and *C. gariepinus* were described as shown in Fig. 3 and 4. Using the first decision rule (Fig. 3), about 99.4% of the erythrocyte of *C. gariepinus* was rounded in shape and only 0.4% was oval cells. In contrast, the erythrocyte of *P. hypophthalmus* had a relatively high proportion of oval shape of 60.2 and 29.8% rounded erythrocyte. Using the second decision rule, however, the proportion of strongly rounded and slightly oval *C. gariepinus* erythrocyte was approximately 1:1 (50.4 vs 49%, respectively), while oval proportion was only 0.4%. The erythrocyte of *P. hypophthalmus* on the other had much more slightly oval erythrocyte compare to strongly rounded erythrocytes (39.2 vs 0.6%, respectively), however, the proportion of strongly oval shape erythrocyte remain higher than the sum of the other groups (about 60.2%).

Comparing the measured and calculated characteristics shows that *P. hypophthalmus* erythrocyte is larger than that of *C. gariepinus* cells (Table 1). However, the mean cell axis ratio was lower compared to the value calculated for *C. gariepinus* (0.90 vs 0.72, respectively).

Table 1: Erythrocyte characteristics of *Pangasianodon hypophthalmus* and *Clarias gariepinus* (n = 500)

Parameters	♀CG×♂CG	♀PH×♂PH	p-value
Cell major axis (μm)	8.58±0.03[b] (6.75-10.80)	10.62±0.04[a] (8.51-14.15)	0.001
Cell minor axis (μm)	7.70±0.02 (6.28-9.27)	7.66±0.03 (4.79-9.14)	0.320
Nuclear major axis (μm)	3.55±0.01[b] (2.97-4.68)	4.12±0.02[a] (3.11-5.82)	0.001
Nuclear minor axis (μm)	3.30±0.01[a] (2.75-3.83)	2.92±0.01[b] (2.13-3.71)	0.001
Cell area (μm^2)	66.23±0.36[b] (43.15-91.35)	81.33±0.40[a] (43.61-106.57)	0.001
Cell volume (μm^3)	269.10±2.27[b] (144.48-429.77)	328.22±2.55[a] (109.49-488.57)	0.001
Nuclear area (μm^2)	11.73±0.05 (8.82-17.74)	12.05±0.09 (7.91-18.93)	0.152
Nuclear volume (μm^3)	20.38±0.14 (13.06-35.27)	18.66±0.22 (9.18-34.56)	0.074
Cell axis ratio	0.90±0.003[a] (0.72-1.00)	0.72±0.003[b] (0.45-0.94)	0.001

Mean in the same row with different superscript differ significantly ($p \leq 0.05$). Numbers are means±standard errors (recorded range)

DISCUSSION

As said earlier, shape discrimination in erythrocyte studies is subject to a lot of human error using only visual observation and no attempt has been made to standardize the process till date. The use of the cell-axis ratio proposed in this study would bring uniformity and accuracy to the assessment of erythrocyte shape in subsequent studies. It is based on the proven assumption that the closer the cell axis ratio is to unity, that more the erythrocyte would be tagged rounded by visual assessment. The first decision rule outline for this protocol is in line with the general observations made on the erythrocyte of some fish evaluated in previous studies using visual observation only[7,18-21]. However, the second decision rule may better describe the erythrocyte observe as erythrocyte were considered strongly rounded when cell axis ratio is as close as possible to unity (between 1-0.90). Hence, certain erythrocyte which could be difficult to classify using the first decision rule can be rightly placed using the second rule. More so, the observation of cell axis ratio of below 0.50 (i.e minor axis less than half of the major axis) makes it imperative to re-classify the erythrocyte into three groups.

Gayatri and Prafulla[22] had earlier wrongly assumed that fish erythrocyte is predominantly oval shape just like their avian and reptilian counterpart. The observation of higher proportion of rounded erythrocytes for *C. gariepinus* and ellipsoidal (oval) for *P. hypophthalmus* however, suggest that different fish species could have predominate different shapes of erythrocyte and in different proportions. Round shape has been reported as the characteristics of the erythrocyte of Common carp *Cyprinus carpio* Linnaeus, 1958 and *C. gariepinus*[7,8,23]. However, the report of Sayed *et al.*[10] on mono-sex Nile tilapia *Oreochromis niloticus* suggest the fish has predominantly oval shaped erythrocytes. There is a paucity of information on the erythrocyte shape and size of *P. hypophthalmus*, hence, the current study may be the first scientific publication on the erythrocyte of the fish despite a long history of its culture. This study suggests that many biometric parameters of *P. hypophthalmus* were significantly larger than that of *C. gariepinus*. Sevinc *et al.*[24] had earlier opined that the gaseous exchange rate in animals with smaller erythrocytes is higher than that of animals with larger erythrocytes. Hence, this may have implication for the oxygen budget needed for physiological activities of the different fish. Fange[25] further opined that erythrocyte size affects the activity of different species because smaller erythrocyte facilitates physiological exchanges by favoring surface to volume ratio (hence active) than what is obtained in larger erythrocytes. This may also explain the reason for the aggressive nature of the *C. gariepinus*, compared to *P. hypophthalmus*.

CONCLUSION

This study has demonstrated a simplified and a more accuracy method of normal erythrocyte shape discrimination in fish using the cell axis ratio. It was observed that erythrocyte parameters of *P. hypophthalmus* are higher than those of *C. gariepinus* and could be possible reason for differences in the activities of the two species.

SIGNIFICANCE STATEMENT

The advantage of this method over visual observation alone is the possibility of accurately estimating the proportion of the different shapes of the erythrocyte available in any fish, rather than an overall generalization that is inaccurate. Although this study made particular references to *P. hypophthalmus* and *C. gariepinus*, it is believed that this method can be applied to many other fish or animal species.

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