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Morphology of erythrocyte and hematological parameters of red tilapia (*Oreochromis* sp.) after challenge with *Streptococcus agalactiae*

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Abstract

The study focused on investigating the morphology of erythrocyte and some hematological parameters (including Hct index, Hb value, RBC count, WBC & thrombocyte count) on red tilapia (*Oreochromis* sp.) infected *Streptococcus agalactiae* before infection (control), five days post-infection and ten days post-infection. The experiment was arranged with two treatments containing control treatment and infection treatment. Each treatment has 3 replications with 3 tanks/treatment (n=10 fish). In this study, the hematological indexes both enhanced while the size of the erythrocyte reduced over five days and ten days of infection. Immature erythrocytes and abnormal erythrocytes are abundantly present in the peripheral blood of infected red tilapia, explaining the decrease in mean erythrocyte size and the RBC count increases.

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Introduction

Tilapia is currently a popular farmed fish around the world; Asia alone contributed 4.2 million tons in 2018, accounting for 68.8% of the world's total tilapia production (FAO 2020, 2020). Red tilapia (*Oreochromis* sp.) - a hybrid between Blue tilapia and Nile tilapia (*Oreochromis mossambicus* x *Oreochromis niloticus)*, is one of the two species of the family Cichilidae most widely cultured in Asia after Nile tilapia (Miao and Wang, 2020). Red tilapia is commonly farmed because of its outstanding advantages such as the relatively short culture period (about six months), good tolerance to a poor stocking environment, high productivity rate and high nutritional values (Mjoun *et al.*, 2010). In Viet Nam, red tilapia is widely cultured in the Mekong Delta in wooden cages that float in the river banks (Boerlage *et al.*, 2017).

The primary constraint for the aquaculture industry is probably disease (Bondad-Reantaso *et al.*, 2005). Every year, fish diseases cause significant losses to aquaculture in Asia and around the world (Subasinghe, 2005). Common tilapia pathogens include *Flavobacterium columnare, Aeromonas hydrophila, Edwarsiella tarda, Ichthyophitirius multifillis, Tricodhina sp., Gyrodactylus niloticus and Streptococcus* sp. (Klesius *et al.*, 2008). Streptococcal disease has become a major problem in tilapia farming. It contributes to severe economic losses, in which two strains of bacteria, *Streptococcus iniae* and *Streptococcus agalactiae,* are the most common. *Streptococcus agalactiae* was first discovered among the populations of rainbow trout farmed in Japan in 1957 and was later isolated and identified as a Gram-positive, non-mobile and non-spore-forming, spherical or ovoid. In addition, it is also a type of bacteria that is catalase-negative and occurs in pairs or chains when grown in liquid media (Amal *et al.*, 2011).

Hematological investigation contributes to the diagnosis of fish health status (Campbell and Ellis, 2007). There have been many studies on hematological parameters of red tilapia infected by bacteria. However, there has been no study about the variation in hematological parameters and erythrocyte size, especially in red tilapia with hemorrhagic disease caused by *Streptococcus agalactiae*.

This study aims to investigate the change of hematological parameters in association with erythrocyte size change after five days and ten days of *Streptococcus agalactiae* infection with a density of 10^6 CFU.mL^{-1}.

Materials and methods

Biological material and experimental design

A total of 60 healthy red tilapia (about 2.5 months of age) were purchased from National Feeding Center for Southern Freshwater Aquaculture located in Tien Giang province. Fish were stocked in a tank with 100 cm × 50 cm × 80 cm (length × width × height) of each.

The tanks were aerated continuously 24 hours a day. The water used was tap water that has been dechlorinated; changed twice a week, each time changing 2/3 of the water and the temperature is checked daily to avoid poor water quality causing stress for the fish.

Streptococcus agalactiae was provided by the Biochemistry - microbiology laboratory of the Ho Chi Minh City University of Education. The original bacteria were unfrozen and cultured in a petri dish containing BHIA (Brain Heart Infusion Agar), incubated at 30°C for 24h. Colonies were transferred to an Erlenmeyer flask containing BHIB (Brain Heart Infusion Borth) then shaken at 150 rpm at room temperature for 24h for proliferation. Bacterial density was determined by a spectral colorimeter at 540 nm. At value, OD=0.125 is equivalent to a streptococcal density of 1.5×10^8 CFU.mL^{-1}.

Fish were randomly distributed in 6 tanks (10 fish per tank), including control treatment and infection treatment (3 tanks per treatment, equivalent to 3 replications). After being brought back from the

hatchery, fish were kept stable for 60 days. Then, fish were challenged with *S. agalactiae* at a density of 10^6 CFU.mL^{-1}. Five days after infection, the first blood sample was collected and the last one was five days next (Nya and Austin, 2009; Farahi *et al.*, 2010; Guo *et al.*, 2012).

Challenge with Streptococcus agalactiae

Fish were infected by the soaking method (Huu Thinh Nguyen *et al.*, 2001). Place 2L of bacterial suspension with a density of 10^7 CFU.mL^{-1} in a tank containing 18L of dechlorinated water, stir well to obtain 20L of bacteria at a density of 10^6 CFU.mL^{-1}. Drop a group of 20 fish into the tank, soak for 60 minutes, then take it out and transfer it to the old tank.

Hematological analysis and determine RBC size

For hematological analysis, 15 fish per experimental unit blood was withdrawn from the caudal vein using syringes with a drop of 10% EDTA. The number of red blood cells (RBC) was counted on the Neubauer counting chamber. The total count of white blood cells (WBC) and thrombocytes was performed through the consumption of Giemsa staining. Hemoglobin index is determined using Sahli hemoglobin. Hematocrit index was determined by blood centrifugation and measurement of red blood cells/plasma sedimentation ratio.

The RBC count was calculated according to (Natt and Herrick, 1952) by the equation:

RBC = A×5×10×200 (cells/mm^3) (A: the total number of red blood cells in the five-count zones).

The total count of WBC and thrombocytes by the equation following (Hrubec *et al.*, 2000):

The total WBC and thrombocyte count = (number of WBC of 1500 cells×R)/1500 (R: the number of red blood cells in 1mm^3 of blood).

Fish RBC size was determined from Giemsa staining slides on the microscope and connected with S-EYE software.

Statistical analysis

The results were submitted to ANOVA one way ($p<0.05$) using Minitab 18. The significant difference between treatments was determined by the Turkey test ($p<0.05$). The mean data were presented as X±SD (means±standard deviation).

Results and discussion

Morphology of erythrocyte

Blood samples of 15 fish were collected and examined for hematological indexes and erythrocyte size in all experiments at five and ten day's post-infection (dpi).

The data in Table 1 show that the erythrocyte size decreases after 5 and 10 days post-infection, in contrast to the hematological parameters investigated.

Table 1. Erythrocyte size (X±SD) of red tilapia at five and ten day's post-*S. agalactiae* infection.

	Control	5dpi	10dpi
Minor axis (µm)	20.46±4.07^a	14.48±2.03^b	13.73±2.39^a
Major axis (µm)	24.23±4.58^a	20.11±2.53^b	18.28±2.38^c
Area (µm)	71.17±9.10^a	55.25±5.54^b	51.03±5.51^c
Perimeter (µm^2)	387.27±99.59^a	229.18±45.50^b	197.31±45.43^c

The erythrocyte size recorded the highest values of minor and major axis in control and gradually decreased over time of infection; the differences were statistically significant ($p<0.05$). Variations in minor and major axis lengths led to similar erythrocyte area and perimeter differences. Thus, the appearance of pathogens (*Streptococcus agalactiae*) in peripheral blood caused erythrocyte size to gradually decrease, but hematological indexes increased.

The increase of immature erythrocytes count in the peripheral blood might explain the decrease in mean

erythrocytes size. In the present study, microscopic observation of blood films showed a greater number of immature erythrocytes than mature erythrocytes (Fig. 1). In peripheral blood smear, immature erythrocytes were identified as round to oval, large N:C (nucleus: cytoplasm) ratio, and smaller than mature erythrocytes (Campbell and Ellis, 2007). In addition, the peripheral blood smear of infected fish also showed abnormal erythrocytes such as teardrop erythrocyte, bean-shaped erythrocyte, burr and binuclear erythrocyte (Fig. 2).

Some studies in diseased fish have also noted an increase in the number of immature erythrocytes and abnormal erythrocytes (Duncan and Lovell, 1994; Miwa and Inouye, 1999).

Table 2. Hematological parameters (X±SD) of red tilapia at five and ten days post *S. agalactiae* infection.

	Control	5dpi	10dpi
Hct (%)	37.55 ± 6.08^{b}	40.65 ± 5.50^{a}	40.81 ± 4.33^{a}
Hb (g%)	8.35 ± 1.12^{c}	9.06 ± 0.99^{b}	10.28 ± 1.00^{a}
RBC ($\times10^{6}$ cell/mm^3)	1.33 ± 0.36^{b}	1.68 ± 0.36^{a}	1.81 ± 0.43^{a}
WBC&thrombocytes ($\times10^{4}$ cell/mm^3)	4.92 ± 2.41^{c}	6.47 ± 2.42^{b}	9.21 ± 2.93^{a}

a, b, c – The difference is statistically significant (p<0.05).

Hematological parameters

The results in Table 2 show that the hematological indexes increase gradually over five days and ten days of bacterial infection, contrary to the erythrocyte size investigated. The changes in hematological indexes are more clearly shown through the Hb value and total count of WBC - thrombocytes. The Hb value in healthy fish reached 8.35±1.12 g%, increased by 8.5% after five days of infection and got the highest value at five days next (10.28±1.00 g%).

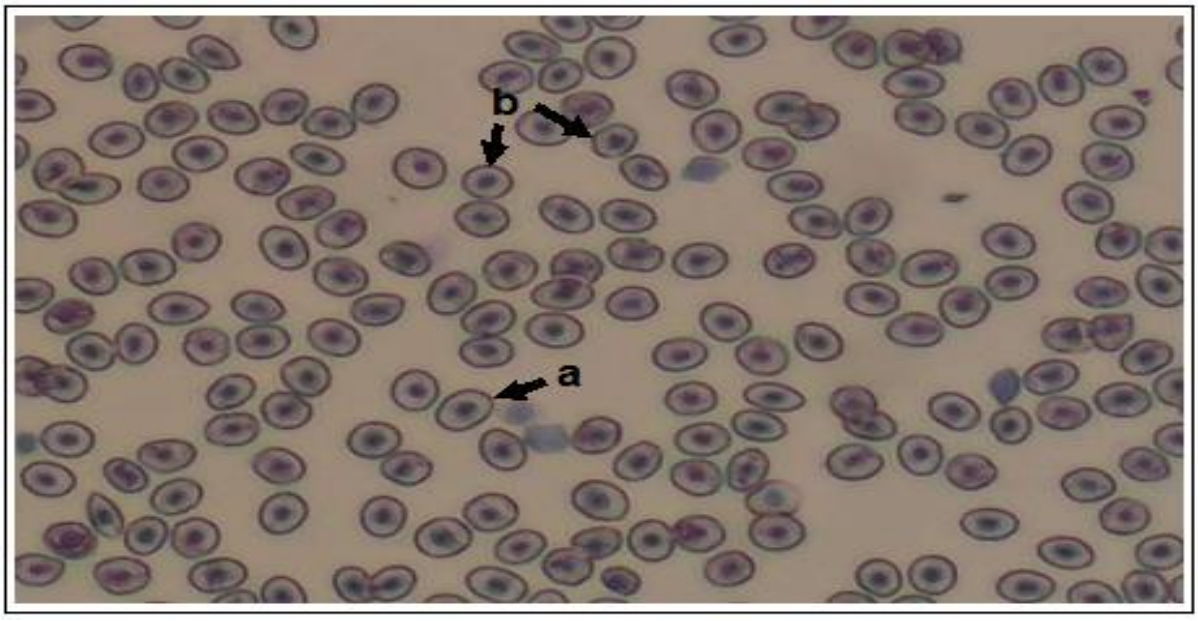

Fig. 1. Mature erythrocyte (a) and immature erythrocyte (b) of infected red tilapia peripheral blood (x20).

The total count of WBC - thrombocytes increased faster through the time of infection. Specifically, this value reached 4.92±2.41 $\times10^{4}$ cell/mm^3 in control, increased by 31.5% at five days post-infection and 42.3% at the next five days. Meanwhile, the Hct indexes and the RBC count increased, but there was no significant difference between 5 days and ten days of infection ($p>0.05$). The study of Alsaid *et al.* (2015) about hematological indexes of red tilapia challenge with *S. agalactiae* at a density of 10^{4} CFU.mL^{-1} showed a decreasing tendency in erythrocyte indexes (including RBC count, Hct indexes (or PCV), Hb

value, MCV, MCH, MCHC) at 1, 3, 5 and 7 days after infection (Alsaid *et al.*, 2015). Similarly, in Nile tilapia *(Oreochromis niloticus)*, Suwannasang *et al.* (2014) infected fish with serotypes Ia and III *Streptococcus agalactiae* (two common serotypes of tilapia). It was reported that both strains caused a decrease in the RBC count during 1-9 days post-infection; the Hb value decreased during 1-3 dpi and returned to normal after six days of infection. The Hct index showed no significant difference between the infected and the control group (Suwannasang *et al.*, 2014). The contrary result was observed in this study when the presence of bacteria in the peripheral blood enhanced erythrocyte indexes. This could be explained that the number of immature erythrocytes increased and gradually replaced erythrocytes because of the influence of stressors such as hypoxia, bacteria, and anemia (Groff and Zinkl, 1999; Clauss *et al.*, 2008).

The impact of bacteria might result in shortened erythrocytes cycle or destroyed erythrocytes which promoted the production of immature erythrocytes (hemolytic erythrocytes) (Duncan and Lovell, 1994).

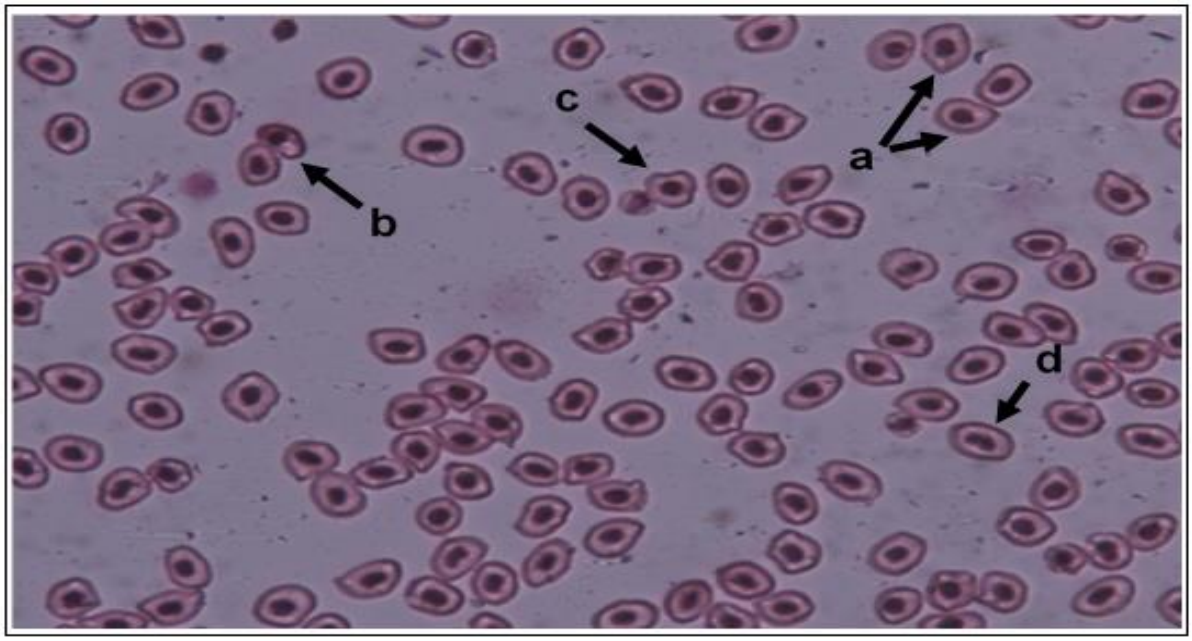

Fig. 2. Peripheral blood smear of infected red tilapia after ten days, showing some teardrop erythrocytes (a), bean-shaped erythrocytes (b), burr erythrocytes (c), binuclear erythrocytes (d) (x20).

White blood cells play an important role in the body's defense against foreign factors by phagocytosis and antibody production. The similarity of the present study with the studies of Suwannasang *et al.* (2014), Alsaid *et al.* (2015) on tilapias infected with *S. agalactiae* is the rapid increase in WBC count after infection. This result is similar to the studies when tilapias infected other bacteria. For example, Tavares-Dias *et al.* (2002) studied Nile tilapia with gill Ichthyophthiriasis and Saprolegniasis observed an increase in neutrophil and monocyte count followed by a decrease in lymphocyte count. Surveying the WBC count in tilapia infected with *Enterococcus* sp., Martin *et al.* (2008) reported opposite results (reduction in neutrophil and monocyte counts, increased the number of lymphocytes). But overall, the mean total of WBC count got a raise. According to the authors, these results demonstrated that fish stimulated by the bacterial infection have their immune system activated (Tavares-Dias *et al.*, 2002; Martins *et al.*, 2009).

Conclusion

This study showed that red tilapia after challenge with *S. agalactiae* at a density of 10^6 CFU.mL^{-1} exhibited increased erythrocyte indexes (including Hct index, Hb value and RBC count) through an increase in the number of immature erythrocytes and the presence of

abnormal erythrocytes in the peripheral blood. Although the result is different from some reported studies because of the combination of many factors, this is also a remarkable result. However, the WBC count increased rapidly over the days of infection, showing similar response to other studies when foreign agents attack.

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