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Pathology of Silver Rasbora (*Rasbora argyrotaenia*) After Experimental Infection with *Edwardsiella tarda*

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Abstract

The high demand for silver rasbora (*Rasbora argyrotaenia*) from the wild has led to declining resources, making fish cultivation necessary. One of the challenges in fish farming is diseases caused by bacteria, such as *Edwardsiella tarda*, which can lead to Edwardsiellosis and result in mass mortality among fish. This study aimed to analyze the pathology of silver rasbora after experimental infection with *E. tarda*. Four hundred silver rasbora (5.4±7 cm and 0.47±2.63 g) were reared in twenty aquariums. The treatments consisted of four groups with five replicates, including immersion in *E. tarda* 10^{11} CFU/ml (P1), 10^{12} CFU/ml (P2), 10^{13} CFU/ml (P3), and a negative control (without *E. tarda*) (P0) for a 14-day rearing period. Parameters observed included survival rate, stress responses (plasma cortisol, blood glucose, and ventilation rate); hematology profile (total erythrocytes, total leukocytes, differential leukocytes, and hemoglobin levels), histopathological alterations in organs, survival rate, and clinical symptoms. The results showed that immersion in *E. tarda* did not affect fish mortality. However, other parameters, including hematological profile, stress responses, and histopathological alterations, showed increased values and several pathological changes, yet remained within normal limits.

INTRODUCTION

Silver rasbora (*Rasbora argyrotaenia*) is a freshwater fish that has high economic potential, but its availability in nature is decreasing due to overfishing and

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environmental contamination (Muchlisin *et al.*, 2010). Fish farming or aquaculture is one of the effective solutions to preserve silver rasbora in wild stock (Budiharjo, 2002). Nowadays, good aquaculture practices are implemented in silver rasbora farming to meet domestic or international market demand for this species. However, the problem of infectious diseases such as *Edwardsiella tarda*, which causes Edwardsiellosis in major freshwater species, is a main obstacle to fish farming activities, leading to high economic losses (Xu and Zhang, 2014). *E. tarda* is an opportunistic, Gram-negative, rod-shaped bacterium that is motile and causes infection and mortality in yellowtail and sea bass (Park *et al.*, 2012), Nile tilapia, African catfish (Nantongo *et al.*, 2019), and climbing perch (Loh *et al.*, 2014; 2023).

Other than aquaculture species, *E. tarda* has been isolated from birds, reptiles, invertebrates, amphibians, and mammals, including humans (Park *et al.*, 2012; Mizunoe *et al.*, 2006). Edwardsiellosis has been reported in freshwater fish in India (Choudhury *et al.*, 2017), Nile tilapia (*Oreochromis niloticus*) (Nagy *et al.*, 2018), yellowtail, Japanese flounder, and red sea bream (Matsuyama *et al.*, 2005); striped catfish (*Pangasianodon hypophthalmus*) (Bera *et al.*, 2020); and turbot (*Scophthalmus maximus*) (Xiao *et al.*, 2008) and causes high losses and fish mortality.

Fish infected with *E. tarda* exhibit atypical swimming behavior, such as spiraling and floating near the water surface (Rodrigues *et al.*, 2019). In addition, several external changes also occur with the *E. tarda* infection, such as hemorrhage in the skin and fin, ulceration, excessive mucous production, and swelling in the abdomen (Butar-Butar *et al.*, 2020). Internally, there are white spots and ascites in the liver, kidney, gill, lymph nodes, and intestine (Xu and Zhang, 2014). Histopathological alterations of Edwardsiellosis in fish display necrosis and degeneration (Hu *et al.*, 2022) in the internal organs of *Catla catla* (Devi *et al.*, 2016), *O. niloticus*, and *Clarias gariepinus* (Ibrahem *et al.*, 2011).

The presence of clinical symptoms is caused by *E. tarda* producing extracellular products (ECP), including dermatoxins, adhesins, flagellin, hemolysin, chondroitinase, collagenase, and protease (Wang *et al.*, 2010). Dermatoxins induce skin tissue damage, allowing *E. tarda* to penetrate and bind to the cell surface using adhesins and flagellin (He *et al.*, 2012). Zhang *et al.* (2016) stated that *E. tarda* invades the host cells, undergoes intracellular multiplication, and hence spreads in the host organs using secretion system type III (T3SS) and VI (T6SS).

Edwardsiellosis is frequently reported in fresh and marine water fish, including in four-finger threadfin (*Eleutheronema tetradactylum*) (Cheng *et al.*, 2024), channel catfish (*Ictalurus punctatus*), blue catfish (*I. furcatus*), channel × blue hybrid catfish (Armwood *et al.*, 2022), spotted sea bass (*Lateolabrax maculatus*) (Hu *et al.*, 2022), and tambaqui (*Colossoma macropomum*) (Reis *et al.*, 2023). In a previous study, silver rasbora has been reported to be infected with Aeromoniasis (Ulkhaq *et al.*, 2023) and Streptococcosis (Nugrahani *et al.*, 2021). To our knowledge, this report represents the first documented case of Edwardsiellosis in silver rasbora. Therefore, this study aimed to determine the clinical symptoms of *E. tarda* in silver rasbora (*R. argyrotaenia*). The results of this study are expected to be the primary data to confirm the infection of Edwardsiellosis in silver rasbora.

METHODOLOGY

Ethical Approval

This research was conducted with the approval of the Faculty of Health, Medicine and Life Sciences, Airlangga University (ethical approval: 244/UN3.1.16/KP/2023).

Place and Time

This study was conducted from June – August 2023 in the Teaching Farm, Faculty of Health, Medicine and Life Sciences, Airlangga University. The hematology analysis was carried out in the Instrumental Laboratory, Faculty of Health, Medicine and

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Life Sciences, Airlangga University. The histological preparation was done in the Histology Laboratory, Faculty of Veterinary, Airlangga University.

Research Materials

The material used in this experiment was silver rasbora (5.4±7 cm and 0.47±2.63g), commercial pellet (Matahari Sakti, Indonesia), *E. tarda* isolate, XLD (Xylose Lysine Deoxycholate) medium (HiMedia, India), neutral-buffered formalin (NBF) 10% (Paraform, Indonesia). The apparatus used in this study were glass aquarium (40×30×40 cm), hemocytometer (Assistant, Germany), haemometer (Superior, Indonesia), blood glucose test strip (Easy Touch, Taiwan), hand tally counter (Joyko, Indonesia), pH meter (Resun, Indonesia), pH indicator paper (Merck, Germany), DO meter (Horiba, Poland) and ammonium/ammonia test kit (SERA, Germany).

Research Design

The research design was conducted using a complete randomized design (CRD) consisting of four treatments, and five replication including immersion with *E. tarda* 10^{11} CFU/ml (P1), 10^{12} CFU/ml (P2), 10^{13} CFU/ml (P3), and a control (P0) (without *E. tarda*). Experimental infection was performed using the immersion method based on the natural infection from opportunistic bacteria (Narwiyani and Kurniasih, 2011).

Work Procedure

Experimental Fish Preparation

Four hundred healthy silver rasbora (5.4±7 cm and 0.47±2.63g) were obtained from the Freshwater Aquaculture Center, Umbulan, Pasuruan, Jawa Timur. Fish were immersed in NaCl 30 ppt for 5 min to eliminate ectoparasites and divided into 20 glass aquariums (20 L) with a fish density of 1 fish/m^3. The fish were acclimatized for three weeks at an optimum temperature of 25-27°C with aeration. The fish were fed twice daily at a rate of 5% of body weight with a commercial pellet (31% protein, 5% lipid, 4% fiber, 12% ash). The optimum water quality parameters were maintained during the rearing periods. During the acclimatization process, the fish did not experience mortality, swam normally, and showed no morphological and physiological abnormalities.

Edwardsiella tarda Culture

Fish pathogen, *E. tarda*, was collected from the Center of Standard Test for Fish Quarantine, Quality Control, and Safety of Fishery Products, Jakarta. The biochemical identification was done using conventional tests, including Gram stain, oxidative-fermentative (OF), motility, indole, H_2S, hemolysin, catalase, and oxidase tests (Kebede and Habtamu, 2016). Bacteria were cultured using XLD (Xylose Lysine Deoxycholate) medium (HiMedia, India) and incubated at 30°C for 24 h. For the subsequent in vivo assay, the bacteria were adjusted using NaCl 0.9% following the treatment concentrations.

Experimental Challenge

The experimental challenge was conducted using the immersion method according to the experimental design. Fish were reared in an indoor room with optimal air circulation and lighting from a lamp until 14 days post-infection. During the rearing period, fish were fed twice daily, 5% of body weight, with the same commercial pellet (Matahari Sakti, Indonesia), and no siphoning was done. In addition, survival rate and clinical signs were observed post-*E. tarda* infection, including behavioral and skin surface changes. All water parameters were maintained in optimum condition (temperature: 26-27°C, pH: 5-7, DO: 8-10, and ammonia: 0-0.25 ppm).

Blood, Organs, and Survival Analysis

Blood samples for hematological observation and stress response were taken from 10% of the experimental fish from each treatment at the end of the rearing period. Firstly, fish were cold-anesthetized at 10-15

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°C until no physical movement was observed, then 0,1 mL of blood was taken using a syringe containing an anticoagulant in the vena caudalis and heart. The blood was stored in a sterile microtube for further hematological analysis, including total erythrocytes, total leucocytes, differential leucocytes, and hemoglobin levels (Witeska *et al.*, 2022), and the stress responses, i.e., plasma cortisol (Sadoul and Geffroy, 2019) and blood glucose levels (Qiao *et al.*, 2012). In addition, the ventilation rate was monitored and recorded every 10 minutes for 1 hour using 2 fish per treatment daily in the morning to determine the stress levels (Flint *et al.*, 2015). Fish survival rate (SR), clinical signs, and water quality observation were measured daily until the end of the infection period.

Histopathological preparation from the kidney and liver of infected fish was carried out post-infection (D14) on 10% of the total population of experimental fish. The fish were dissected, and the organs were kept in 10% neutral-buffered formalin (NBF) and processed based on the standard histopathological procedures (Slaoui and Fiette, 2011).

Data Analysis

All data obtained was tabulated using MS Excel 2017 and compared with the optimum value. Meanwhile, histopathology changes were analyzed descriptively in the figure.

RESULTS AND DISCUSSIONS

Hematology Profile

The hematology profile (Table 1) presents a total of erythrocytes (1.06 – 2.29 $\times 10^6$ cells/mm^3) and hemoglobin levels (5.0 – 6.9 g/dL) in all treatments, including the control group. The values were still within normal conditions (1.05 – 3.00 $\times$ 10^6 cell/ mm^3 and 5.00 – 7.06 g/dL, respectively). However, the total leucocyte counts and differential leucocyte percentages displayed higher values than normal, especially the percentage of neutrophils, which increased to twice (68 - 86%) compared to the normal condition (33.62 – 39.12%) and the negative control (38 - 39%).

Table 1. Hematological profiles and stress responses of silver rasbora (*Rasbora argyrotaenia*) after immersion with *Edwardsiella tarda* for 14 days.

Parameters	Treatment				Optimum range
	P0	P1	P2	P3	
Hematology Profile					
Erythrocyte (x10^6 cell/mm^3)	1.06-1.27	1.51-1.94	1.43-2.16	1.38-2.29	1.05-3.0
Hemoglobin (g/dL)	5-5	5-5.2	5.5-6.2	6.7-6.9	5-7.06
Leucocyte (x10^4 cell/mm^3)	8.32-8.56	9.92-10.04	12.6-13.97	12.58-12.59	6.11-9.7
Neutrophil (%)	38-39	68-70	72-87	85-86	33.62-39.12
Monocyte (%)	28-30	7-25	9-16	7-9	29.12-30.81
Lymphocytes (%)	32-33	5-25	4-12	5-8	31.4-33.26
Stress Responses					
Cortisol plasma (ng/ml)	15.24 – 17.38	21.27 – 24.05	31.35 – 38.87	34.8 – 46.1	40-60
Blood glucose (mg/dL)	43.56 – 53.44	71.56 – 81.44	70.31 – 88.69	74.73 – 90.27	40-90
Ventilation rate (bit/10min)	1333.27 – 1629.27	1466.82 – 1765.22	1546.17 – 1695.33	1555.43 – 1693.77	1100-2000

P0 (control/without immersion with *E. tarda*); P1 (10^{11} CFU/ml); P2 (10^{12} CFU/ml); P3 (10^{13} CFU/ml)

The hematological profile of infected *R. argyrotaenia* showed a normal value for all treatments, including control (Table 1). Hematological observation can be used to determine the fish health status and early detection of infectious diseases in fish (Witeska *et al.*, 2022). Based on the result, erythrocytes and hemoglobin are still in the normal range of Cyprinid (Neelima *et al.*, 2015). Its cause is that the *E. tarda* strain

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produces γ-hemolysin based on biochemical characterization (unpublished data). Eissa *et al.* (2016) stated that γ-hemolysin toxin has a weak ability to hemolyse erythrocytes and hemoglobin. Furthermore, Moustafa *et al.* (2016) report that γ-hemolysin has low hemolysis activity and is thermolabile, therefore, it affects bacterial pathogenicity.

Meanwhile, leucocytes demonstrate an increasing value compared with the normal condition, particularly in P2 (immersion with *E. tarda* 10^{12} CFU/ml) and P3 (10^{13} CFU/ml). Leukocyte cells act as the primary fish's defense system against pathogens and also as a sign of infection in the fish's body. The increase in total leucocyte cells indicates the pathogen's invasion of the fish's body, triggering the granulocyte and agranulocyte cells to eliminate the infections (Nourshargh and Alon, 2014). Similar results have also been described in koi carp (Rajapakshe *et al.*, 2012) and catfish (A'yunin, *et al.*, 2020a). Further observation in differential leucocytes shows the highest presentation of neutrophils in P3 (immersion with *E. tarda* 10^{13} CFU/ml) than the normal value. Buchmann (2022) reported neutrophils as the first cells of leucocytes that respond to pathogens and migrate from fish hematopoietic organs to the infected tissue. Furthermore, neutrophils can phagocytize using pseudopodia and also produce several antimicrobial substances, including hydrolytic enzymes and lysozymes, to destroy the pathogens (Zhao *et al.*, 2017).

Stress Responses

Stress responses (Table 1) showed all parameters (plasma cortisol, blood glucose, and ventilation rate) in P2 (immersion with 10^{12} CFU/ml *E. tarda*) and P3 (10^{13} CFU/ml *E. tarda*) as being within normal conditions. Nevertheless, the values in P1 and the control group were lower than in normal conditions. The normal values were 40-60 ng/ml, 40-90 mg/dL, and 1100-2000 bit/10 minutes for plasma cortisol, blood glucose, and ventilation rate, respectively.

Biological stressors in the water, including pathogens, can cause stress in fish (Ackerman and Iwama, 2001). Petitjean *et al.* (2019) classified fish's stress responses to bacterial infection as primary (production of cortisol hormone in the blood), secondary (increase in blood glucose), and tertiary (behavioral changes and increasing ventilation rate). Responses to stress in silver rasbora (Table 1) demonstrate that all parameters, including cortisol plasma, blood glucose, and ventilation rate, were at normal values in fish. These indicate that being immersed in *E. tarda* bacterial suspensions causes no stress and has no influence on their immune system. The normal values of cortisol plasma, blood glucose, and ventilation rate in fish were 40-60 ng/ml (Ming *et al.*, 2012); 40-90 mg/dl (Sulmartiwi *et al.*, 2013); and 1100 – 2000 bit/10 minutes (Flint *et al.*, 2015), respectively.

Histopathological Alteration

Histopathological alteration in the liver (Figure 1) and kidney (Figure 2) illustrates hydropic degeneration and necrosis of different severity. The most severe condition was demonstrated in P3 (10^{13} CFU/ml *E. tarda*) among all treatments.

Histopathology examinations can be used as indicators to illustrate fish health conditions (Rašković *et al.*, 2013). Internal organ changes can occur as a result of an *E. tarda* infection, including in the liver (Rubianto *et al.*, 2016) and the kidney (Meidiza *et al.*, 2017). Histopathology alteration in the liver (Figure 1) and kidney (Figure 2) of silver rasbora after being challenged with *E. tarda* demonstrates hydropic degeneration and necrosis. Hydropic degeneration occurred because cells were unable to maintain ion and fluid balance, limiting ionic pump performance at the plasma membrane. Whereas necrosis is the death of cells or tissue that is accompanied by irreversible degeneration (Andriyanto *et al.*, 2009). These alterations are caused by the synergistic activities of CAH (cell-associated hemolysin) and the

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elastase enzyme secreted from *E. tarda*. The cytotoxic effect of CAH affects organ damage (Darwish *et al.*, 2000). Furthermore, CAH and siderophores produced by *E. tarda* can induce bacterial replication in the circulatory system, absorbing iron nutrients (Igarashi *et al.*, 2002) and causing foci necrosis in internal organs (Mathew *et al.*, 2001).

Sahoo *et al.* (2000) reported similar findings in the climbing perch (*Anabas testudineus*) where those infected with Edwardsiellosis showed histopathological characteristics of liver necrosis, enteritis, and slight necrosis in the spleen and kidneys. Another study in catfish (*Pangasius* sp.) also reported that fish developed hyperplasia and lamella fusion in the gills, necrosis, and edema in the kidneys, and necrosis and vacuolization in the spleen after infection with *E. tarda* (A'yunin *et al.* 2020b). Ewarsiellosis in African catfish (*Clarias gariepinus*) fingerlings exhibited necrosis in the nephritic tubule, hemosiderin deposition, and edema in the kidney. As well as focal necrosis, inflammation, and lymphocyte infiltration in the liver (Abraham *et al.*, 2015).

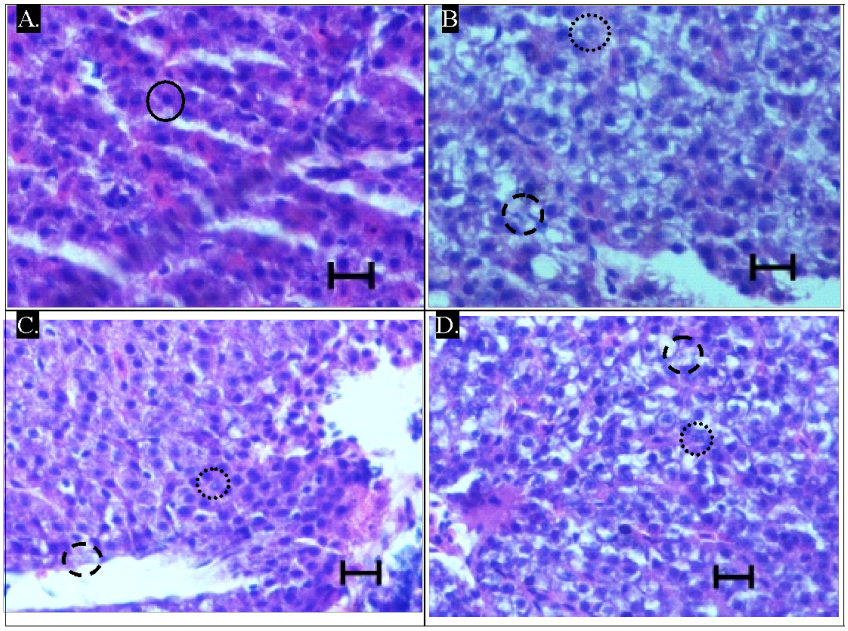

Figure 1. Liver histopathology of silver rasbora after being challenged with *E. tarda* for 14 days (HE staining).

Description: (A) control/without immersion with *E. tarda*, (B) immersion with *E. tarda* 10^{11} CFU/ml, (C) 10^{12} CFU/ml, (D) 10^{13} CFU/ml. ····· = Hepatocyte ,—— = Degeneration,----- = Necrosis, Scale bar = 50μm.

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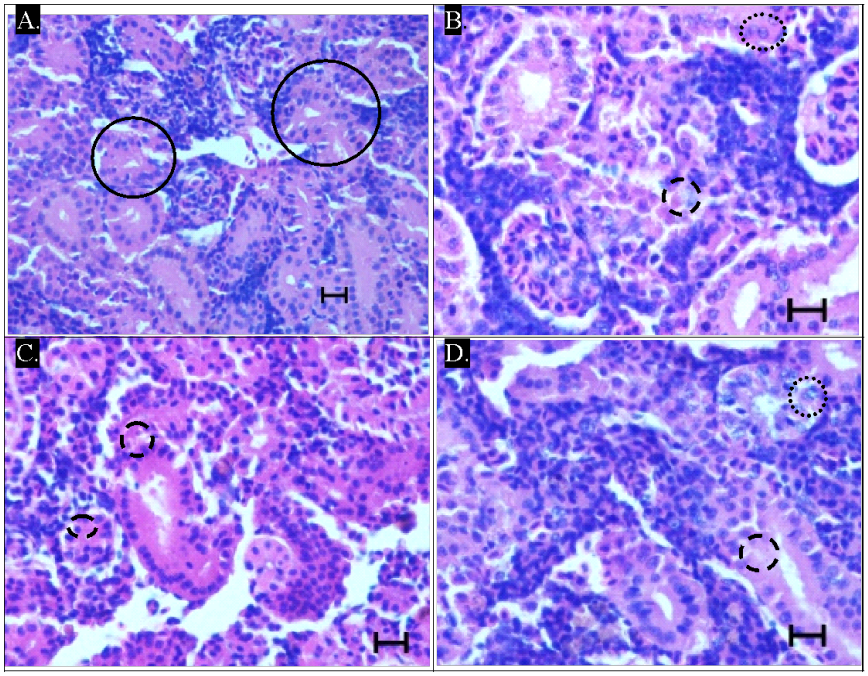

Figure 2. Kidney histopathology of silver rasbora after challenged with *E. tarda* for 14 days (HE staining).

Description: (A) control/without immersion with *E. tarda*, (B) immersion with *E. tarda* 10^{11} CFU/ml, (C) 10^{12} CFU/ml, (D) 10^{13} CFU/ml. ── = Tubulus, ▪▪▪▪ = Degeneration, ----- = Necrosis, Scale bar = 50μm.

Survival Rate and Clinical Sign

Silver rasbora mortality after immersion with *E. tarda* for 14 days (Figure 3) indicates that in all treatments were no mortality was observed. Fish were in normal condition, no behavioral and no external or internal alterations.

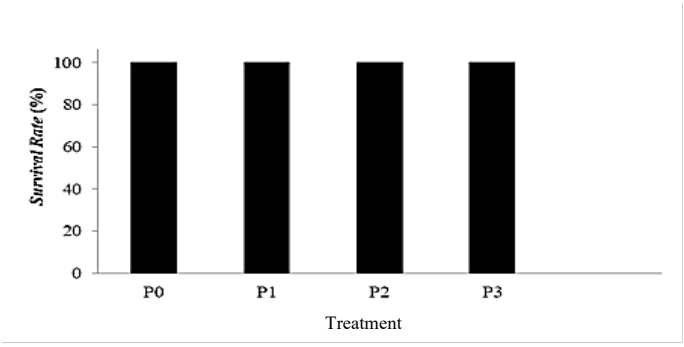

Figure 3. The survival rate of silver rasbora (*R. argyrotaenia*) after being challenged with *E. tarda* for 14 days.

Description: (P0) control/without immersion with *E. tarda*, (P1) immersion with *E. tarda* 10^{11} CFU/ml, (P2) 10^{12} CFU/ml, (P3) 10^{13} CFU/ml.

The clinical signs (Figure 4) demonstrate no changes after being challenged with *E. tarda*. Fish showed silver skin and normal condition (Figure 4a), normal abdomen (Figure 4b), and dorsal (Figure 4c).

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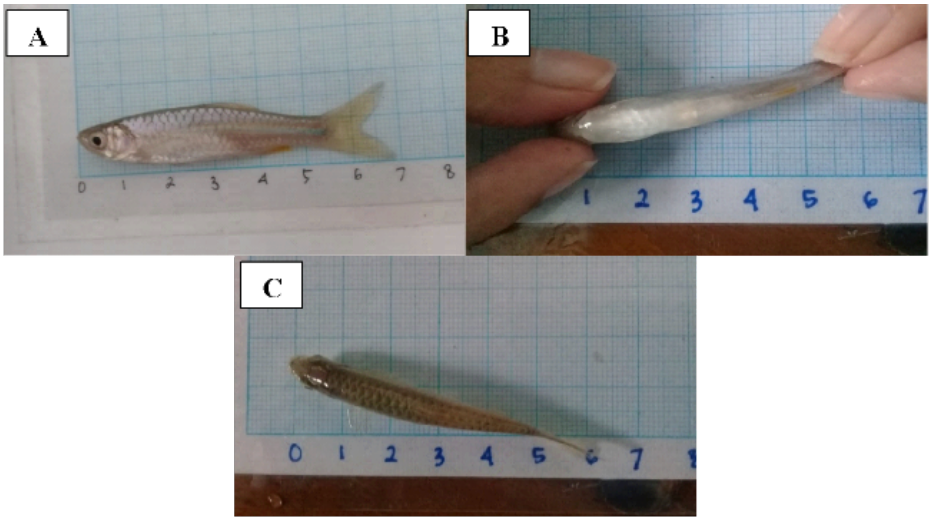

Figure 4. Silver rasbora condition after being challenged with *E. tarda* for 14 days.
Description: (A) silver body laterally; (B) normally in the abdomen; (C) normally in dorsal.

Survival (Figure 3) and clinical signs (Figure 4) observation in infected silver rasbora with *E. tarda* for 14 days present no mortality and gross clinical signs appeared. Abraham *et al.* (2015) reported that catfish fingerlings with Edwardsiellosis infection demonstrated abnormalities in swimming behavior and external alterations, including vertical hanging, overproduction of mucus, abdominal swelling, and fin and tail deterioration. Furthermore, Algammal *et al.* (2022) also found a swollen abdomen, reddish operculum, and hemorrhages in *O. niloticus* after being naturally infected with *E. tarda*. This condition suggests that *E. tarda* has not been available, causing disease in silver rasbora despite entering the blood and internal organs.

Husna *et al.* (2022) reported the viability of *E. tarda* in the blood, liver, and kidney of silver rasbora ranging from 1,9 – 8,8 × 10^4 CFU/ml, below the minimum quorum of *E. tarda* (3 × 10^7 CFU/ml) (Zhang *et al.*, 2009). The bacterial minimum viability must be accomplished (quorum sensing) to trigger infection and disease by communicating amongst cells via extracellular signaling molecules (autoinducers). Quorum sensing (QS) can increase the bacterial amount as well as impact the activity and virulence of bacterial pathogens in the host (Rutherford and Bassler, 2012).

CONCLUSION

The pathology of silver rasbora after experimental infection with *E. tarda* densities reaching 10^{13} CFU/ml has not been able to cause mortality, alteration of hematological profiles, stress responses, and histopathology alterations.

CONFLICT OF INTEREST

There is no conflict of interest between the authors in writing and publishing this manuscript.

AUTHOR CONTRIBUTION

The author's contribution consisted of: IAN, MF, ARR, MFU: Conceptualization, Investigation, Methodology, Supervision, Validation, Writing-Original Draft Preparation, Writing-Review & Editing; WT, RK: Conceptualization, Methodology, Supervision; MAP, DPK, JYL: Formal Analysis, Validation, Writing-Review & Editing.

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