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Immunohistochemical Studies of *Aeromonas Salmonicida* Infection in Freshwater Pomfret (*Colossoma Macropomum*)

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Abstract

Aeromonas salmonicida cause furunculosis disease in both freshwater and marine fish, which represents a great threat in fish farming. The research aimed to develop and produce polyclonal *A. salmonicida* antibody from rabbits to strengthen diagnostic method of furunculosis through immunohistochemistry method. A total of 15 samples of freshwater pomfret (*Colossoma macropomum*) was divided into 3 groups which intramuscularly injection with 10^9 cell/mL of *Aeromonas salmonicida* antigen, Euthanasia was done on the third, sixth, and ninth day post infection to take organs for histological and immunohistochemical process. Polyclonal primary antibody against *A. salmonicida* infection was obtained from rabbit's serum which was previously immunized with the O antigen of *A. salmonicida* containing 10^9 cells/mL, with weekly increased volume (0.5; 1; 2; 3 mL). Anti-*Aeromonas salmonicida* rabbit serum was then purified to obtain its immunoglobulin G by precipitating it first using ammonium sulfate and filtered using dialysis membrane. Immunoreactivity against the bacterial antigen was evidenced in the skin, spleen, liver, and kidney starting from third day post infection and further distributed until ninth day post infection. Distribution of bacteria in the fish was evidenced by histology examination characterized by vascular and necrotic changes with immunohistochemistry staining. Polyclonal antibody against *Aeromonas salmonicida* and immunohistochemistry was found to be useful for identifying the pathogen from the first stage of the disease and differentiate *A. salmonicida* infection from others bacterial septicemic disorders in freshwater pomfret.

Keywords: *Aeromonas salmonicida*; freshwater pomfret; immunohistochemistry; polyclonal antibody

Introduction

Aeromonas is a normal microbiota which commonly found in water. In heavy infection and immunosuppressed fish, *Aeromonas salmonicida* may cause furunculosis disease in both freshwater and marine fish. Few virulence factors possessed by *Aeromonas* species include aerolysin, cytotonic and cytotoxic enterotoxins, protease, and DNAse is known to induce the invasion of the bacteria and pathogenesis of bacterial spreading within the host's body. The clinical signs and pathological changes of *A. salmonicida* infected host is not specific due to its similarity with other bacteria, such as *Aeromonas hydrophila* and *Streptococcus iniae*, with furunculosis as the main clinical sign. Noga (2010) found that fish infected by *A. salmonicida* subsp. *salmonicida* was not always showed furunculosis as its typical clinical signs. In non-salmonid fishes, manifestation of this pathogenic bacteria showed to be ulcerative dermatitis. Hiney at al. (1994) and Figueras (2005) reported that detection in *A. salmonicida* infected fish with asymptomatic furunculosis was

become the important factor of disease spreading, as the fish become carrier of this pathogenic bacteria. Within the carrier fish, *A. salmonicida* subsp. *salmonicida* play an important role as the opportunistic pathogen, which only affects stressed or immunocompromised fish.

Identification of *Aeromonas* strain using classic method is done by characteristic differentiation of every species, this method is said to be ineffective by Beaz-Hidalgo et al. (2010) due to the bias result and similarity with one and another species, for example *A. hydrophila* and *A. sobria* is difficult to be distinguished as both of this bacteria showed positive results in arginine dihydrolase test and both of them were able to hydrolyze glutamine. Another similarity was found in *A. salmonicida* and *A. hydrophila* as they were β -hemolytic and glucose-fermented bacteria. Gold standard method to identify *Aeromonas sp* strain according to Kusumawaty et al. (2016) is by 16s rDNA analysis. This method is known to be accurate and very sensitive for differentiating every single *Aeromonas* species, otherwise, this method takes long time and highly depends on DNA sequencing. Coscelli et al. (2014) developed immunohistochemistry method to detect *Aeromonas salmonicida* in turbot with furunculosis as the main revealed clinical sign. The result of his study showed that immunohistochemistry method specifically proved to detect *A. salmonicida* antigen within the host tissue and it was beneficial for pathogenesis study of acute furunculosis.

The research aimed to develop and produce polyclonal *A. salmonicida* antibody from rabbits to strengthen diagnostic method of furunculosis in freshwater pomfret through immunohistochemistry method.

Materials and Methods

Two male New Zealand rabbits weighed 2 kg were apparently healthy without detectable signs and symptoms of any disease. They were acclimatized for one week under controlled condition in the experimental animal house of the Department of Pathology, Faculty of Veterinary Medicine, Gadjah Mada University, providing appropriate feed, water, and well maintained proper hygienic condition. However they were immunized by intraperitoneal injection with the O antigen of *A. salmonicida* containing 10^9 cells/mL, with weekly increased volume (0.5; 1; 2; 3 mL). Five weeks after immunization, the blood sample was taken from each rabbit and kept in slanted position for one hour at room temperature. After the blood became clotted, the antiserum was collected by a pipetter in centrifuge tubes. After centrifugation at 1000 rpm for 15 minutes, the serum was separated from the red blood cells and heated at 58°C for half an hour to inactivate the complements. Such prepared complement inactivated anti-*Aeromonas salmonicida* rabbit serum was then purified to obtain its immunoglobulin G by precipitating it first using ammonium sulfate.

A total of 15 samples of freshwater pomfret (*Colossoma macropomum*) was divided into 3 groups: A, B, and C, each group consisted of 5 fishes intramuscularly injected with 10^9 cell/mL *A. salmonicida* antigen from previous production as much as 0.1 mL. On the third day post infection, all fish from group A was sacrificed. The organs obtained from each fish were gill, skin, heart, liver, spleen, intestine, and kidney. The same procedure was done in group B and C on sixth and ninth day post infection.

The organs such as gill, skin, heart, liver, spleen, intestine, and kidney on day 3, 6, and 9 post-injection were fixed in 10% neutral buffer formalin solution for histhological process.

Tissue sections of fish were de-waxed and rehydrated. All incubation steps were performed at room temperature in a humid chamber and slides were washed with 10 mM phosphate-buffered saline (PBS), pH 7.4, in two successive immersions of 5 minutes between every step. Endogenous peroxidase was quenched by Peroxidase Blocking Solution (Leica) for 30 minutes. Tissue sections were incubated for 30 minutes with the affinity-purified rabbit anti-*A. salmonicida* (anti-*A. salmonicida*, diluted 1:250), washed and then incubated for 30 min with an anti-rabbit EnVision + System Labelled Polymer-HRP (Leica). Sections were developed employing 3,3 diaminobenzidine tetrahydrocloride as chromogen (Leica). Finally the immunolabelled tissue sections were counterstained with haematoxylin. The slides were observed by light microscope to get a positive reaction of *A. salmonicida antibody.*

Results

Significant gross lesions were not observed in challenged fish as the main clinical sign is furunculosis which seen as furuncles in the skin and/or muscle, progressing to crater lesions followed by darkening of the body color. Experimentally infected freshwater pomfret did not show any significant gross lesions such as furuncle on its skin, this might because of chronic and acute furunculosis occur and generally depends on water temperature, age of the fish and pathogenicity of the strain of *A. salmonicida*. Fish may die acutely with few or no prior signs of disease and limited pathological changes, as the fishes were sacrificed in the third to ninth day which may still categorized as acute stage disease. In the third day post infection of *A. salmonicida* antigen, the epidermis layer of intramuscular injection site showed strong immunolabeling suggested the accumulation of *A. salmonicida* antigen which reacted with the anti-*A. salmonicida* polyclonal antibody (Figure 1 A). Following the bacterial invasion to the deeper part of the skin to reach the bloodstream and other target organs, in the sixth day post infection (Figure 1 B) the antigen of *A. salmonicida* was detected in the epidermal-dermal part of the skin until it finally detected the muscle part (Figure 1 C) of the skin in the sixth day post infection.

The antigenic distribution occur rapidly as the systemic infection plays an important role, involving heart as the main organ of circulatory system. Positive reaction was observed in the pericardial layer starting from the third day post infection with *A. salmonicida* antigen (Figure 2 A). The bacteria further spread to the myocardial layer in the sixth and ninth day post infection (Figure 2 B, 2 C).

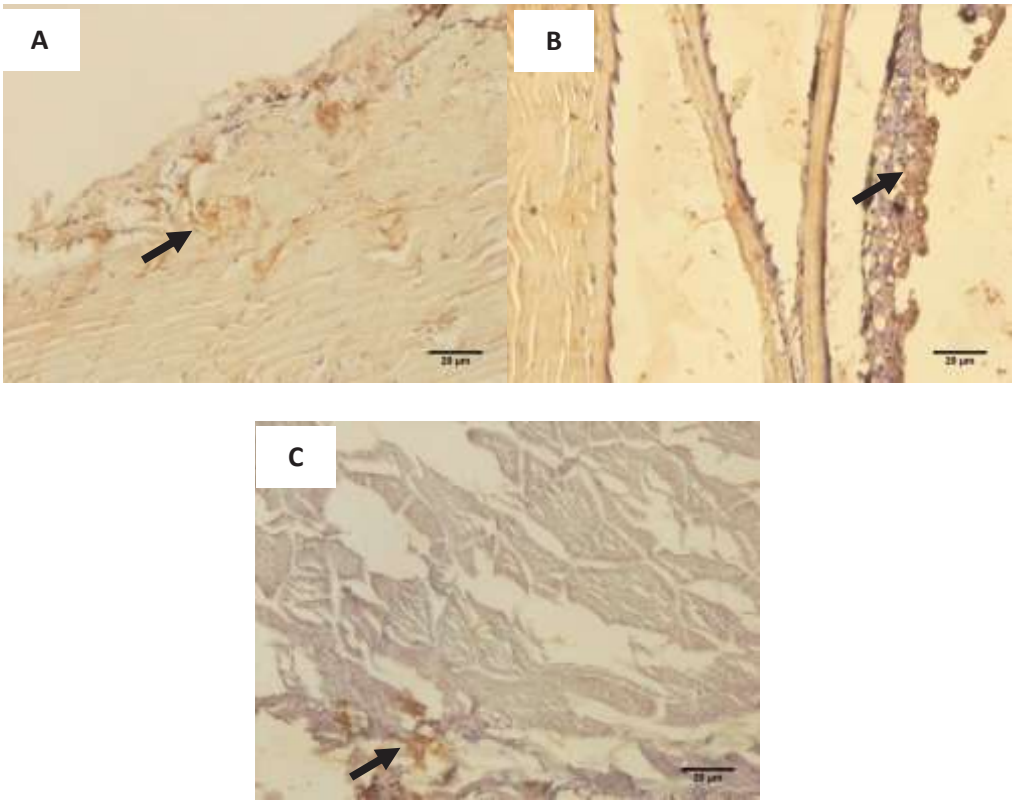

Figure 1. Skin layer immunohistochemistry photomicrograph of freshwater pomfret (A) third, (B) sixth, and (C) ninth day post infection (arrow) (IHC 20x).

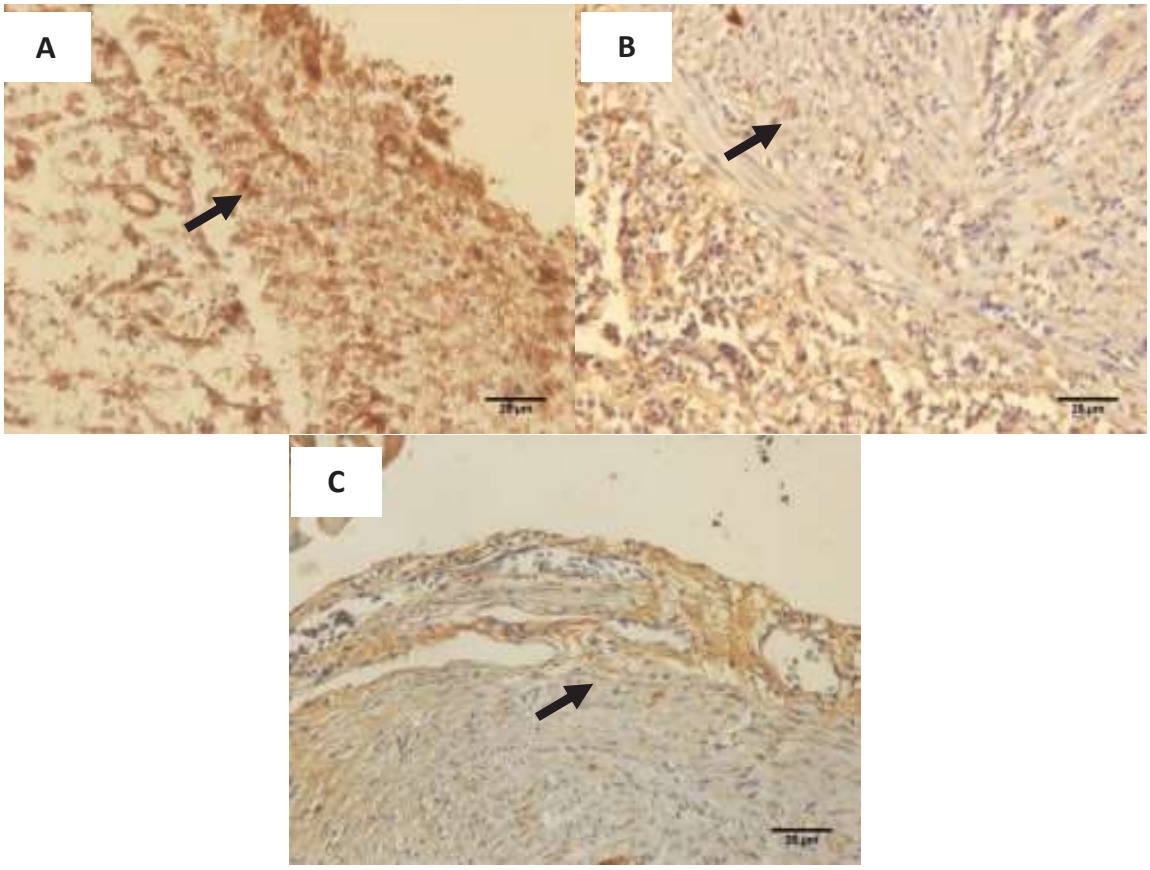

Figure 2. Pericardial layer immunohistochemistry photomicrograph of freshwater pomfret (A) third, (B) sixth, and (C) ninth day post infection (arrow) (IHC 20x).

The immunohistochemistry imaging of spleen regarding septicemia were clearly evidenced in the sixth day post infection with *A. salmonicida* antigen, within the ellipsoidal sheath of the infected spleen showed positive immunolabeling (Figure 3 A) and continue spread ninth day post infection (Figure 3 B).

The immunohistochemistry imaging of kidney evidenced in the third day post infection with *A. salmonicida* antigen, showed positive immunolabeling (Figure 4 A) and because of the bacteria continued to multiply, in the sixth day post infection showed that the positive immunolabeling was spread in the renal tubules (Figure 4 B). The

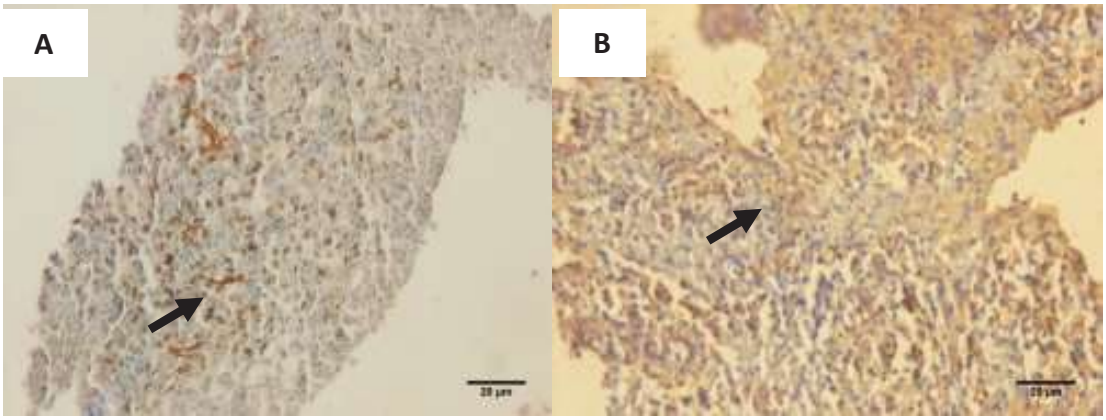

Figure 3. Immunohistochemistry photomicrograph of spleen freshwater pomfret (A) third, (B) ninth day post infection (arrow) (IHC 20x).

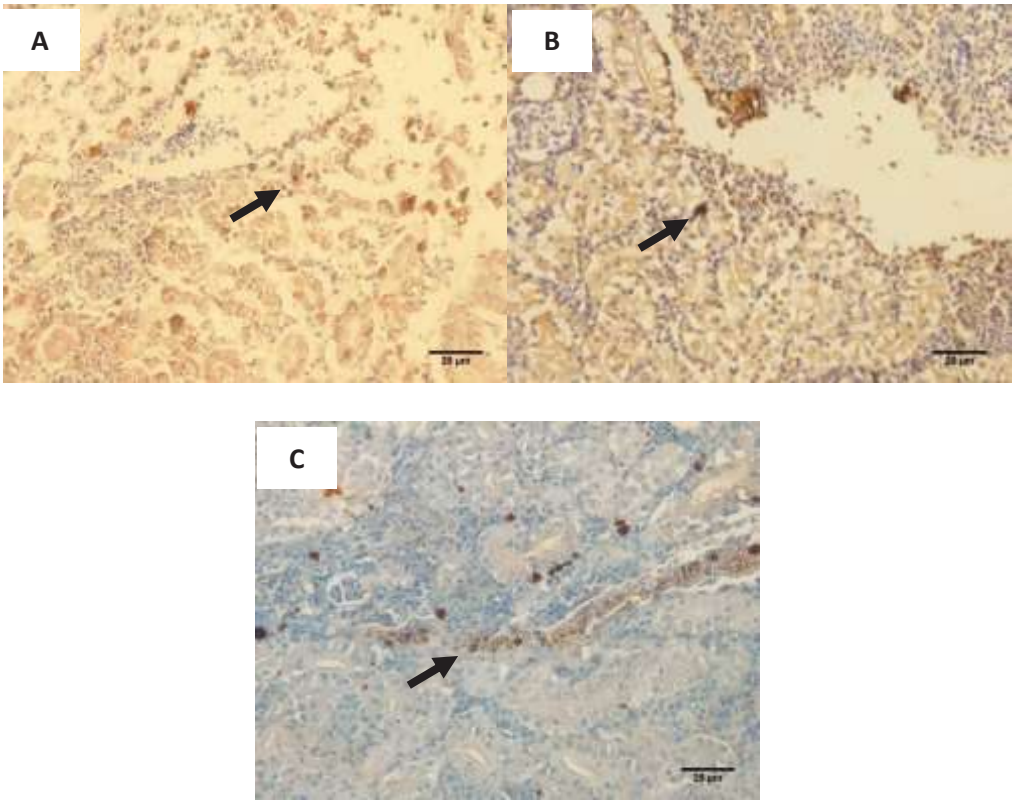

Figure 4. Immunohistochemistry photomicrograph of kidney freshwater pomfret (A) third, (B) sixth, and (C) ninth day post infection (arrow) (IHC 20x).

septicemia continued as the interstitial tissue which contained blood vessel was also showed positive immunolabeling in the ninth day post infection (Figure 4 C).

Pathological findings showed that intramuscular injection of *A. salmonicida* in freshwater pomfret resulted in a septicaemia compatible with the acute form of furunculosis described in salmonids as previously studied. Immunohistochemistry detection of *A. salmonicida* antigens demonstrated the rapid blood distribution of the bacteria to target organs and the development of the lesions. The immunostaining of the bacteria in the pericardium third day post infection indicates that *A. salmonicida* rapidly reaches the bloodstream to the heart which may help the bacteria spreads systemically to the other target organs. Previous study was done by Farto et al. (2011) also showed that *A. salmonicida* was detected in several internal organs from 2 to 12 hours post bath challenge infection in Atlantic salmon and turbot.

The significant histological lesions of *A. salmonicida* infection consisted on vascular congestion, widespread haemorrhages as well as necrosis of tissues and accumulation of inflammatory cells affecting several organs. Microscopic lesions showed under light microscopy were inflammation and congestion of gills (Figure 5 A). Pericarditis as showed by the accumulation of neutrophil in the pericardium (Figure 5 B). The liver showed congestion and vacuolization in the hepatocytes (Figure 5 C). The kidney showed necrosis in the tubules and the accumulation of melanomacrophages (Figure 5 D), the spleen also showed haemorrhage (Figure 5 E), and inflammation occurred within the submucosal layer of the intestine of infected fish (Figure 5 F).

Figure 5. Histopathology of freshwater pomfret infected with *A. salmonicida* (A) congestion of gill (arrow), (B) accumulation of inflammatory cells within pericardium (arrow), (C) liver congestion (arrow), (D) necrosis of the renal tubules, (↑) accumulation of melanomacrophage center (arrow). (E) spleen haemorrhage. (F) accumulation of inflammatory cells within intestine mucosal layer (↑)

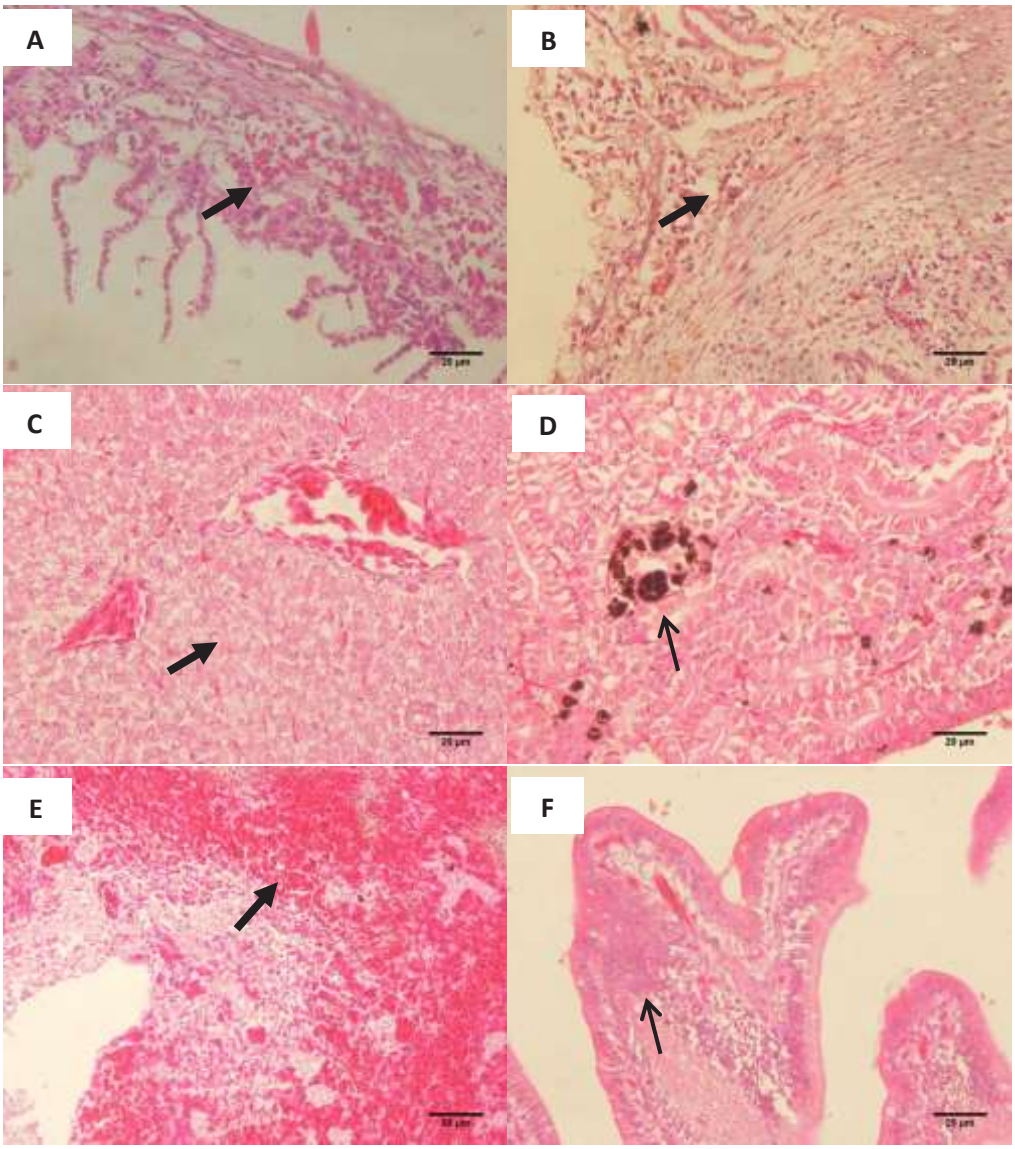

Figure 5. Histopathology of freshwater pomfret infected with A. salmonicida (A) congestion of gill (arrow), (B) accumulation of inflammatory cells within pericardium (arrow), (C) liver congestion (arrow), (D) necrosis of the renal tubules, (↑) accumulation of melanomacrophage center (arrow). (E) spleen haemorrhage. (F) accumulation of inflammatory cells within intestine mucosal layer (↑)

The vascular disturbance as shown in the figures were congestion occurred in the gill and liver, whereas the haemorrhage occurred in spleen is thought to be caused of the toxin released by *A. salmonicida* such as haemolysin which acts against fish erythrocytes. The mobilization of inflammatory cells and multiplication of the bacteria following the vascular disturbances are compatible with systemic inflammatory response. These were similar to previous studies of salmonid fish infected with *A. salmonicida* (Bjornsdottir et al., 2005; Noga, 2010; Alyahya et al., 2018).

The immunohistochemistry imaging is related with the histopathological imaging of heart in the sixth day post infection which the septicemia was started to develop as the bacteria started to spread and accumulate in the vascular system, including heart as the main vascular organ, causing inflammation at the site of accumulation and the antigen of the bacteria was stained brownish as the result of antigen-antibody reaction which was visualized by DAB chromogen.

The visualization of *A. salmonicida* antigen associated with blood vessels and necrotic areas supports a strong relationship between lesions and the presence of bacteria. Histology of experimentally infected fish showed necrosis in the renal tubules and the immunoreactivity of the

tissue with the antibody is positively strong. Tissue necrosis probably resulted from cellular hypoxia due to vascular damage, as well as direct damage caused by the action of toxins and enzymes secreted by *A. salmonicida* (Orozova et al., 2009; Lago et al., 2012; Vanden Bergh et al., 2013).

Conclusion

Antigen of *Aeromonas salmonicida* was detected in the skin and hematopoietic organ starting from third day post infection infection. Polyclonal antibody against *Aeromonas salmonicida* and immunohistochemistry was useful for identifying the pathogen from the first stage of *A. salmonicida* infection from others bacterial septicemic disorders.

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