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Detection of *Aeromonas hydrophila* infection on Tilapia fish (*Oreochronis niloticus*) skin by Hematoxillin-Eosin and immunohistochemistry staining

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Abstract

Aeromonas hydrophila infection has caused major losses in fish industries in Indonesia. An accurate diagnosis of the diseases caused by *A.hydrophila* is needed in order to increase the effectiveness of the treatment. The aim of this research was to observe the histopathological changes in Tilapia fish skin caused by *A.hydrophila* infection. Five *A.hydrophila*-infected tilapia fish skin samples with hemorrhagic and ulceration lesions were made into histopathologic specimen by using H-E staining. Immunohistochemistry staining was applied on the skin samples by using primary antibody obtained from rabbit and secondary antibody with HRP-System. Afterwards, IHC staining was done to further confirm the diagnosis of *A.hydrophila* infection. The histopathological findings of *A.hydrophila* on Tilapia fish skin tissue samples were focal hemorrhagic lesions and infiltration of neutrophils. All 5 tissue samples were tested positive for *A.hydrophila* in the immunohistochemistry staining, which indicated brown color on the tissue. This research concluded that the histopathological changes in *A.hydrophila*-infected fish skin included hemorrhages and neutrophils infiltration. The diagnosis was further confirmed by the brown color indicated in the immunohistochemistry staining.

Keywords: *Aeromonas hydrophila*; Hematoxylin-Eosin; Immunohistochemistry; Neutrophils infiltration

Introduction

One of the common disease outbreaks that affect tilapia fish is Motil Aeromonas Septicemia (MAS) disease caused by *Aeromonas hydrophilia* (Rahmaningsih, 2012). Motil Aeromonas Septicemia, commonly known as red spot disease, was first declared as an epidemic in Indonesia in 1980, and caused the death of 82.2 tons of fish in 1 month (Angka et. al., 1982).

According to a study by Cipriano et. al., 2001, the clinical symptoms of MAS disease in tilapia include hemorrhagic and ulcerative skin lesions. Furthermore, according to research by Laith and Najiah, 2013, the histopathological lesions observed on the skin of *A.hydrophila*-infected catfish were necrosis in the dermal layer. Since MAS lesions are not specific, IHC staining is usually performed as a means to diagnose MAS disease. Nasution et. al., 2015, succeeded in using the IHC technique to detect *Coxiella burnetti* antigen in cattle organs. In order to determine the histopathological changes on fish infected with *A.hydrophila*, histopathological observations of the skin tissue of *A.hydrophila*-infected fish should be made after the HE staining.

The differential diagnosis of MAS fish includes Edwardsiellosis, Furunculosis, Bacterial Kidney Disease, Viral Hemorrhagic Septicemia and Gyrodactylosis (Anonymous, 2017). In order to find more effective therapy and preventative measure against MAS disease, the diagnosis will be confirmed by immunohistochemical staining (Matos et al., 2010).

This study aims to obtain the histopathological picture of tilapia skin which is infected with *A.hydrophila* by using the HE staining method and to confirm the diagnosis of the disease caused by *A.hydrophila* through immunohistochemical staining.

Materials and Methods

The present study analyzed 5 samples of Tilapia skin tissue which had been diagnosed with *A. hydrophila*, Other materials used in this study were primary antibodies from rabbit serum which had been injected with bacterial isolates, 10% Buffer Neutral Formaldehyde (BNF), *A. hydrophila* isolate, Hematoxylin-Eosin dye, Dako Envision + Dual Link System-HRP (K4065. USA), 3-3.Diaminobenzidine (DAB) chromogen, xylol solution, ethanol, Phosphate Buffer Saline (PBS), Tween-20, citric buffer, 0.3% H2O2 in methanol, Fetal Bovine Serum (FBS) and entellan. The equipment was a set of necropsy tools, a set of tissue processing tools, glass objects, deck glass, coating slides, micropipettes, cassettes, microscope, water bath and microtome.

Preparation of fish skin histopathology

The histopathological preparations were made according to the method of Carson (1990). A ± 1 cm cut was taken from the skin where lesions/wounds were found. Then, the skin tissue was fixed in 10% Buffer Neutral Formaldehyde (BNF) for 24 hours. The next step was the trimming process which was conducted by cutting 5 x 5 x 3 mm of the skin tissue in the cassette. After that, the dehydration process was carried out in several stages by using ethanol solution in concentrations of 70%, 80%, 90% and absolute ethanol for 60 minutes each. Then, xylol solution was added three times for 60 minutes each and a clearing process was carried out, followed by the process of infiltrating paraffin into the skin tissue twice for 60 minutes each. The next procedure was the embedding process. In this process, the tissue was printed on top of the paraffin block and cut ± 5 µm by using a microtome. The tissue piece in the paraffin block was then put into a water bath and placed in a glass slide for the drying process.

Immunohistochemical staining

The immunohistochemical staining was carried out according to Carson's (1990) method. Immunohistochemistry was carried out using polyclonal antibodies against Aeromonas sp., which was harvested from optimized and diluted rabbit antisera. Afterwards, secondary antibodies and chromogen 3-3, diaminobenzidine (DAB) (Dako Envision + Dual Link System-HRP (K4065, USA) was added by following a procedure which consisted of placing the tissue slide on an object glass, conducting the deparaffinization process in a xylol solution (3 repetitions, 3 minutes each), conducting the rehydration process in a multilevel ethanol solution (absolute ethanol for 3 minutes in two repetitions, 95% for 3 minutes in two repetitions, 80% for 3 minutes, and 70% for 3 minutes), and washing the tissue with phosphate buffer saline or PBS (3 repetitions, 5 minutes each). Then, the antigen retrieval method was carried out on the tissue slide by immersing it in a citrate buffer at 90 °C-95 °C for 15 minutes, washing the tissue slide with PBS (3 repetitions, 5 minutes each). Next, endogenous activity blocking was performed at 0.3% H2O2 in methanol for 30 minutes, and then the tissue was washed using PBS + Tween 20 (3 repetitions for 5 minutes each). After that, the tissue slides were incubated in serum with the addition of 1% Fetal Bovine Serum (FBS) for 30 minutes, and incubated in polyclonal antibody against Aeromonas for 24 hours. Then, the tissue slides were washed with PBS + tween 20 in 3 repetitions for 5 minutes each. Afterwards, secondary antibody (Dako Envision + Dual Link System-HRP, K4065, USA) was administered for 30 minutes, and the tissues were washed with PBS + tween 20 (3 replications, 5 minutes each). Next, the substrate or chromogen 3-3, diaminobenzidine (DAB) was added for 2-5 minutes, then the tissue slides were washed in distilled water. The counterstain was performed using a Hematoxylin solution for 10 minutes. The tissues were then washed with running water for 5 minutes, followed by a dehydration process with gradually increasing alcohol concentration (80%, 95%, and absolute alcohol for 3 minutes each). Next, the clearing process was performed in xylol solution (3 repetitions, 3 minutes each). Finally, the slides were covered with a glass object coated

with entellan and then observed under a binocular microscope (Olympus DP12).

Hematoxylin-Eosin Staining

After the deparaffinization process, xylol solution was added twice for 3 minutes each. Then, the fish skin tissue was put in absolute ethanol, 80% ethanol and 70% ethanol for 2 minutes each for rehydration. Afterwards, the tissue was soaked in Hematoxylin solution for 10 minutes. Then, the tissue was rinsed with distilled water four times and rinsed with running water for 15 minutes. Next, the tissue was immersed in Eosin solution for 10 minutes followed by 70% ethanol, 90% ethanol, then absolute ethanol for 1 minute for the dehydration process. After that, the tissue was cleared using xylol solution three times for 2 minutes. Then, entellan was added to the tissue and the slide was closed with a glass object. The histopathological data, which include inflammatory cell infiltration, hemorrhage and tissue cell damage, was analyzed descriptively.

Result Analysis

The results of the immunohistochemical staining on the fish skin were analyzed descriptively and qualitatively. A positive result was indicated by a brownish color reaction on the tissue. It is an indication of the presence of antigen and antibody bonds from Aeromonas sp. on the examined tissue or organ. The results of organ staining using HE were analyzed by observing pathological changes in the fish skin.

Results and Discussion

Observation of the histopathological changes in the five samples of tilapia skin was carried out microscopically using histopathological preparations with HE staining. Observation of *A. hydrophila* antigen in fish skin tissue was carried out by immunohistochemical staining. Research data are presented in tables and figures. Table 1 shows the macroscopic and microscopic changes of the five tilapia samples.

The observation on the five tilapia samples that were diagnosed with *A.hydrophila* infection indicated macroscopic changes in the form of hemorrhage and ulceration. Microscopically, there was an infiltration of neutrophil cells and hemorrhage on the skin which were clearly visible through HE staining.

Table 1: The results of macroscopic and microscopic changes observed in 5 tilapia infected with *A.hydrophila*

Sample	Macroscopic symptoms	Microscopic symptoms
Tilapia-1	focal hemorrhage at the lateral side of the body	neutrophil infiltration, hemorrhage
Tilapia-2	ulcer, skin necrosis at the lateral side of the body	neutrophil infiltration
Tilapia-3	hemorrhage at the lateral side of the body	hemorrhage, neutrophil infiltration
Tilapia-4	hemorrhage at the abdomen	hemorrhage, neutrophil infiltration
Tilapia-5	hemorrhage at the caudal side of the body	hemorrhage, neutrophil infiltration

The histopathological changes of the third tilapia fish skin showed hemorrhage and neutrophil infiltration as seen in Figure 1.

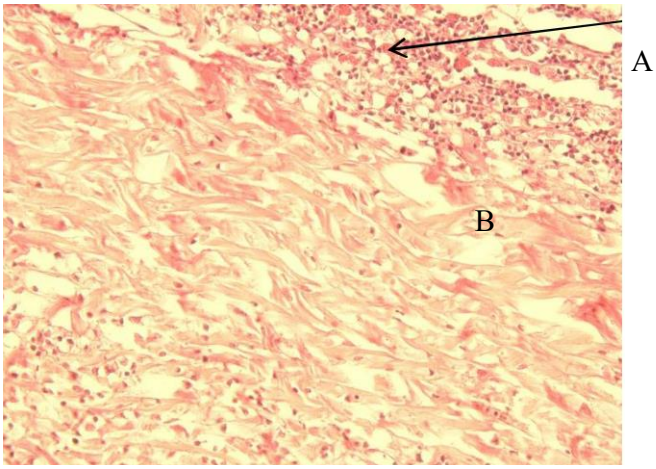

Figure 1. The histopathological image of the third tilapia (*Oreochromis niloticus*) skin sample showing a) hemorrhage in tilapia epidermis and b) neutrophil infiltration in tilapia epidermis. (H&E staining, 400x magnification)

These histopathological changes were the same as the changes found by Cipriano (2008). Another histopathological change is necrosis of the dermal tissue of the fish. (Laith and Najiah, 2013) The virulence factor of *A.hydrophila* that causes the pathological changes is aerolysin. Aerolysin is a type of β-haemolysins produced by *A.hydrophila* during the exponential phase of the bacterial growth. Aerolysin is thermo-stable (56 °C for 5 minutes), and is a toxin that forms

pores causing osmotic lysis and total destruction of erythrocytes (Liu, 2015). Laith and Najiah (2013) also argue that lipase and hydrolipase are important virulence factors of *A. hydrophila* as they change the structure of the host cell cytoplasmic membrane.

Macroscopic changes found in the five tilapia were hemorrhage and ulceration, which are also reported by Hirono and Aoki (1991), in the form of focal hemorrhage and ulceration of the skin. According to the investigation by Cipriano (2001), chronic *A. hydrophila* infection often causes dermal ulceration, focal hemorrhage and inflammation. Furthermore, according to Hanson et al. (2012), changes in acute *A. hydrophila* infection are exophthalmia, skin hemorrhage and ascites.

The diagnosis of *A. hydrophila* infection on fish skin was further confirmed by performing immunohistochemical staining on the fish skin tissue. Immunohistochemistry is a method of staining using substances or active ingredients in tissues based on the basic principle of immunology, namely the binding of a specific antigen with specific antibodies. Histopathological findings of *A. hydrophila* in the fish skin appear as brown marks resulting from the interaction between the antigen that binds to the primary antibody and the secondary antibody conjugated with HRP (Horse Radish Peroxidase) and DAB chromogen which helps form a brown color after being oxidized by peroxidase enzymes in secondary antibodies. (Bintari, 2016) The results of IHC staining on the five tilapia infected with *A. hydrophila* are seen in Table 2.

Table 2: IHC results of 5 tilapia fish skin samples which are infected with *A.hydrophila*

Sample	Immunohistochemical result
Tilapia-2	Positive, brown color distributed mostly in the dermis
Tilapia -3	Positive, brown color distributed mostly in the dermis
Tilapia -4	Positive, brown color distributed mostly in the dermis
Tilapia -5	Positive, brown color distributed mostly in the dermis

The results of IHC staining of healthy tilapia fish skin compared to tilapia skin that are infected with *A.hydrophila* are shown in Figure 2.

The primary antibody was made from rabbit serum injected with *A.hydrophila* antigen. All of the samples showed a brownish color as the result of IHC staining, so it can be concluded that there was a positive reaction with the *A. hydrophila* antigen. The histological result of the control tilapia skin showed a slightly brownish color in the epidermis layer even though there is no *A. hydrophila* infection. It happened because *A. hydrophila* is a normal flora in the fish tissue, and infection will occur only if the fish's immune is weakened (Hanson et. al., 2012). The results of IHC staining also indicated that the *A. hydrophila* antigen was mostly distributed in the dermis of the tilapia fish skin.

Conclusion

From the findings, it is concluded that *A.hydrophila* causes histopathological changes in the form of hemorrhage and neutrophil infiltration

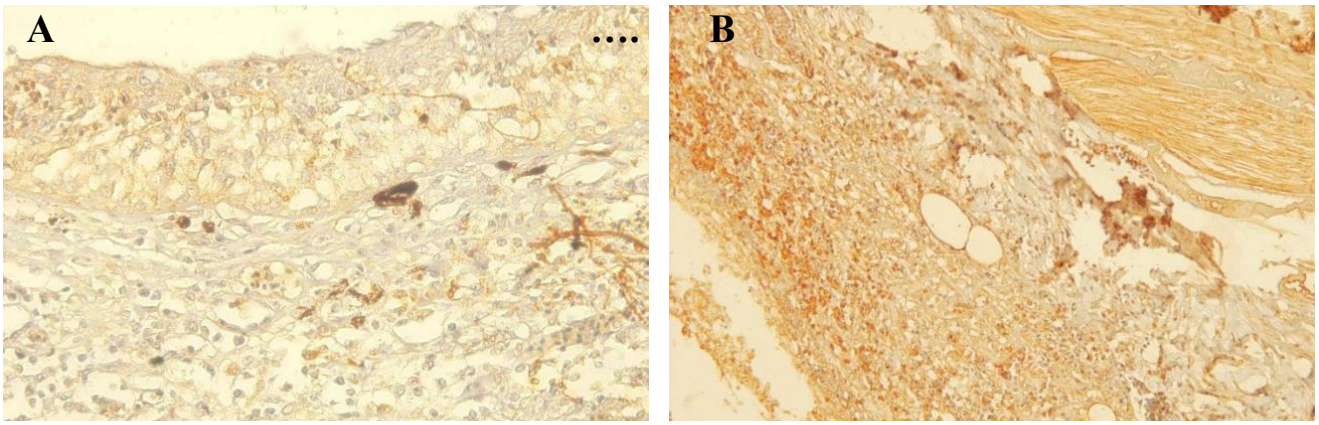

Figure 2. A. Histological image of negative control tilapia skin after IHC staining, 200x magnification. B. Histological image of the skin of Tilapia-3 infected with *A.hydrophila* after immunohistochemical staining, 20x magnification. The bond between the antigen and the antibody is visualized with a brownish color.

that can be observed by using HE staining in the skin tissue of tilapia (*Oreochromis niloticus*). The positive reaction resulting from the interaction between *A. hydrophila* antigen and the primary antibody from rabbit could be observed as brownish color on the dermis of the fish skin as shown by the immunohistochemical staining.

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