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ANALYSIS OF THE PATHOGENESIS OF *Aeromonas hydrophila* IN THE AFRICAN CATFISH, *Clarias gariepinus* AND INVOLVEMENT OF THE TNF- α IN RESPONSE TO THE INFECTION

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(Received: May 30, 2022; Final revision: June 24, 2022; Accepted: June 24, 2022)

ABSTRACT

This research aimed to study the pathogenesis of *Aeromonas hydrophila* infection through two different routes of infection in African catfish and to find out the involvement of TNF- α in response to the infection. The experimental infection model was performed by clipping the caudal fin and immersing the fish in a medium with *A. hydrophila* and by intramuscular injection. Total plate count were used to investigate total and the distribution of *A. hydrophila* in the organs and TNF- α were observed using immunohistochemistry. The results showed that the two types of infection were able to show typical *A. hydrophila* symptoms in experimental fish. Histological observation indicated that the two types of experimental infection resulted in systemic aeromoniasis infection. Total bacterial count results showed that *A. hydrophila* were detected three hours post-infection (hpi) in all organs, except for the kidney, in which detection started since hour 0, both in control and challenge fish. TNF- α were detected in all experimental fish and influenced by the number of bacteria, the function and tissue structure of the organs. It can be concluded that artificial infection by clipping the caudal fin of *Clarias gariepinus* and immersing the fish in a medium with active *A. hydrophila* isolates cause systemic aeromoniasis infection in organs. Acute infection with *Aeromonas hydrophila* causes an increase in TNF- α production.

KEYWORDS: *Aeromonas hydrophila*; histology; immunohistochemistry; TNF- α ; *Clarias gariepinus*

INTRODUCTION

Increased production in catfish (*Clarias gariepinus*) farming is encouraged to meet the increasing food needs of the community, but this is often inhibited by several factors, one of which is the disease. The economic loss due to this disease is worth one billion US dollars every year (Assefa & Abunna, 2018). One of the opportunistic bacteria attacking aquaculture is *Aeromonas hydrophila*, which is responsible for motile aeromonas septicemia (MAS). *A. hydrophila* causes a high level of mortality in aquaculture across the world and wide spread economic losses (Jiang *et al.*, 2017), including in catfish farming. In the United States, it was reported that the economic loss caused to catfish aquaculture by MAS epidemic attack stood around \$60-70 million (Shoemaker *et al.*, 2018).

The MAS disease generally spreads horizontally through direct contact with the water or diseased fish, aquaculture intensification with high stocking density, and lowered water quality, stress will increasing the risk of *A. hydrophila* infection. Wounds due to abrasion between fish in aquaculture with high stocking density or incorrect handlings may open up a gateway for pathogens to enter the body, spread, and cause septicemia (Zhang *et al.*, 2016). An artificial infection model that mimics natural infection involving bath in water medium and making artificial wounds on the fish body provides information on the factors that can increase susceptibility to *A. hydrophila* infection (Zhang *et al.*, 2016).

Variations in the pathogen entrance portal such as skin lesion, gastro intestinal tract, or injection will have a role in the differences in pathogenesis. Information on pathogenesis in infections resembling natural infections or infections with pathogen injection into the fish body will be useful in disease control, especially for rapid detection, treatment, and

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prevention. Proper handling of *A. hydrophila* infection will prevent high mortality rates, use of chemicals that may harm the environment, and residue that may influence the quality of fishery products.

One of the components of the innate immune system involved in pathogenic infections is the pro inflammatory cytokine *tumor necrosis factor alpha* (TNF- α) which is both antimicrobial and immunomodulatory (Hong *et al.*, 2013; Zhang *et al.*, 2012; Kadowaki *et al.*, 2009; Roca *et al.*, 2008). TNF- α has a role as a mediator in the resistance to parasitic, bacterial, and viral diseases (Czarniecki, 1993; Wellmer *et al.*, 2001). The knowledge of the cellular composition and production of cytokines along with histological analysis may be helpful in the understanding of pathogenic mechanism, immune evasion, and determination of appropriate artificial infection model and test animals, thereby allowing clear understanding of the detailed mechanism in *A. hydrophila* pathogenesis. Such information will eventually facilitate development of vaccines and therapeutic drugs for *A. hydrophila* infection in catfish aquaculture (Nya & Austin, 2011). Immunohistochemical assays can give information on the distribution of the immune system's effector cells in tissue after a bacterial infection and its relation to clinical symptoms, pathologic changes in tissue, and distribution of bacterial cells after infection that is important to know. The aim of this research was to study the pathogenesis of *A. hydrophila* infection through two different pathogen entrance points and to detect the kinetics of a TNF- α -expressing leucocyte population in catfish.

MATERIALS AND METHODS

Fish and Experimental Condition

A total of 660 catfish, *Clarias gariepinus* 11.81 \pm 0.47 cm and 9.87 \pm 0.22 g in measure were obtained from a fish farmer in West Java. The fish were reared for two weeks prior to being used in testing and acclimatized for two weeks in a controlled tank (temperature 28.56°C \pm 0.27°C, pH 7.46 \pm 0.11, and dissolved oxygen 5.92 \pm 0.41 mg L⁻¹). Every week, the water was changed at 30% and syphoning was performed to remove the food residue and dropping. Koch's postulate test was performed on the isolate to confirm the disease role and conducting pathogenicity test.

Bacteriology

A. hydrophila (MH3), was obtained from collection of the Freshwater Aquaculture and Fisheries Extension, Depok, BRPBATPP, Sempur, Indonesia. Bacterial

culture was performed on tryptic soy agar (TSA) medium for an incubation time of 24 hours, from which a separated colony was then extracted to be grown in 10 L of liquid medium (tryptic soy broth/TSB). The *A. hydrophila* culture in the liquid medium was incubated in an incubator shaker for 24 hours at room temperature. The culture was then washed three times in saline by centrifugation at 4,000 rpm for 10 minutes.

A. hydrophila Experimental Infection

The experimental fish were assigned into four groups, each consisting of 24 fish, and replication was performed three times. Artificial infection was conducted in two ways. First, it followed the modified procedure developed by Zhang *et al.* (2016) and Reimchen & Temple (2004). This infection method was performed by making uniform wounds by clipping \pm 80%-85% of the caudal fin. After the clipping of the caudal fin by a pair of scissors, the fish were assigned into two groups. In group A, the fish were immersed in water medium with active *A. hydrophila* isolates at 2 x 10⁶ cfu/mL in TSB for two hours. In group B, the fish were immersed in water medium without addition of *A. hydrophila* isolates for two hours. The second method of artificial infection was intramuscular (IM) injection of 0.1 x 10⁸ cfu/mL active *A. hydrophila* isolates (group C). In control group, fish injected with 0.1 mL of sterile PBS.

Clinical symptoms were observed each day, the organs of three experimental fish i.e kidney, liver, intestine, muscle, and spleen were collected at 3, 6, 24, 48, and 96 hours post-infection (hpi). Sampling was carried out randomly in both fish with obvious clinical symptoms and fish without any specific clinical symptoms. Total plate count assay (Madigan *et al.*, 2011) and serial dilution was performed according to the experiment dosage.

Histology

Tissues were fixed in 4% buffered paraformaldehyde for 3 x 24 hours before transferring to 70% ethanol for histopathologic and immunohistochemical preparation. Fixed tissues were carried out by routine histological processing and embedded in paraffin wax. Sections of 5 μ m were deparaffinized, stained with standard method of Mayer's Haematoxylin and Eosin (H&E). The severity of infection was determined by the score of pathological abnormalities of *A. hydrophila* infection based on Grizzel & Kiryu (1993) (Table 1). Histopathological examinations by using light microscope (Olympus Life Science) with x40-100 magnifications.

Table 1. Level of severity in the kidney, liver, spleen, muscle, and intestine infected by *A. hydrophila* (Modified from Grizzle & Kiryu, 1993)

Level of severity	Description	Diagnosis
0	No necrosis; cells appear normal	
1	Necrotic cells rarely occur, and if there are any, they spread; there are some lymphocytes (10 locations); there is a little change in the sizes of the cell nuclei (a minimum of 6 cell nuclei); the atrophic cell quantity increases: 10 cells (5 cells in each of 2 fields).	Limited infection of wounds on the skin and in the muscle showing an early symptom or the fish's resistance to the infection
2	Monofocal necrotic cells are widespread; the central venous sinus appears widened due to mild swallowing; lymphocyte/macrophage count increases, appearing evenly spread; whole cell count and cell thickness from 10 visual fields start to decrease; cell nuclei appear to change in size (a minimum of 6 cell nuclei); the atrophic cell quantity increases: 10 cells (5 cells in each of 2 visual fields); macrophages are filled with melanosomes.	Latent infection toward systemic aeromoniasis infection
3	There are extensive, multifocal necrotic cells; the central venous sinus is widened significantly due to swallowing/ inflammation; lymphocyte/macrophage count is very high; whole cell count and cell thickness significantly decrease; there is a significant change in cell nuclei (a minimum of cell nuclei); the atrophic cell quantity increases: more than 10 cells (more than 5 cells for each of two visual fields); the number of macrophages filled with melanosome increases; cells appear to undergo hyperplasia; cell nuclei appear enlarged significantly	Systemic aeromoniasis infection

Immunohistochemistry

Primary antibodies for immunohistochemical assay to detect the protein signal of TNF- α in tissue section was rabbit polyclonal anti TNF- α antibody (dilution 1:600) (Abcam, UK). Immunohistochemical protocols based on Adam & De Mateo (1994), and Coscelli *et al.* (2016) with modification. Tissue sections were dewaxed in xylene and rehydrated through graded alcohol. The deparaffinized tissue section were incubate with 3% H₂O₂ in ethanol for 30 minutes. The tissues were incubated in 0.2% trypsin in CaCl₂ for 120 minutes at room temperature for antigen retrieval. After that, sections were incubated with 10% normal serum in 30 minutes at 37°C for non-specific

protein blocking. The sections were incubated with the primary antibody all night long at 4°C; then, the sections were incubated with anti-rabbit biotinylated antibody as secondary antibody, for 30 minutes at 37°C. And then, re-incubated with streptavidin-horse-radish peroxidase. Every turn of stage, rinsing with PBS was performed three times. Between different steps, slides were washed with 10 mM phosphate-buffered saline (PBS) 0.5% Tween 20, pH 7.4, in three successive washes of five minutes. Immunostaining was performed with diaminobenzidine (DAB) chromogenic substrate kit. In the staining test using DAB, the result would be declared positive (+) if from microscopy a brownish color was observed.

Qualitative and quantitative analysis of TNF- α in tissue following the previously procedure describe by Coscelli *et al.* (2016) and Ronza *et al.* (2015). Observation was conducted using a light microscope (Olympus Life Science) with x40-100 magnifications. The number of positive cells was counted in all images using imaging software Image J.

Statistical Analysis

The non-parametric Mann-Whitney *U* test (MW) was used to evaluate the difference between infected and non-infected fish group at each sampling point, and difference between sampling points within each experimental group were compared using the Kruskal-Wallis H test (KW) followed by Dunn test. Analyses were performed using the statistical software package IBM SPSS Statistics 20 (SPSS Inc.). The accepted level of significance for the test was < 0.05 .

RESULTS AND DISCUSSION

Clinical and Macroscopical Findings

In group with PBS steril injection, fish fed normally and no clinical signs or mortality was observed during the experiment period same as group of clipped at caudal fins without immersed *A. hydrophila* isolate, no inflammation around the clipped caudal fin regions and did not exhibit significant symptomatic differences (Figure 1a). In contrast, after experimental infection of *A. hydrophila* fish showed inflammatory responses and continued to exhibit abnormal swimming behavior and food intake both clipped caudal fins with emersion and IM-injection treatment. Fish developed hemorrhages and muscular lesions around the clipped caudal fins in bath group (Figure 1b). Infected group with IM-injection, hemorrhages and muscular lesions were observed at the injection sites (Figure 1c). Mortality started at 24 hpi in both

of infected group and reached a cumulative mortality of 47.77% and 51.11% at 72 hpi in groups of *A. hydrophila* bath infection and IM-injection, respectively (Figure 2). Both of infected fish exhibit abnormalities such tender and yellowish liver, pale, and swollen spleen and haemorrhage in kidney.

The results of this study indicate that experimental infection of catfish by clipping the caudal fin of *C. gariepinus* and immersing the fish in a medium with active *A. hydrophila* isolates can cause *A. hydrophila* infection such as infection by IM-injection. Experimental infection by such method is considered similar to natural infection. Clipping the caudal fin induces stress that predisposes the fish to infection (Zhang *et al.*, 2016). Furthermore, clipping the caudal fin is a method of making uniform wounds as the entrance points of pathogens as with the water-borne pathogen route in natural infections (Zhang *et al.*, 2016).

The clinical symptom of the *A. hydrophila* experimental infection in *C. gariepinus* that were infected both through artificial wounds and through IM-injection was alike to the clinical symptom of *A. hydrophila* stated by Hal & El Barbary (2020) and Janda & Abbott (2010). Some factors reported to have caused an increase of *A. hydrophila* pathogenicity are long term incubation, bacterial concentration, and environmental factors such as water quality and temperature (Yambot, 1998). Other factors are feed residue, low salinity, the presence of *A. hydrophila* carrier such as bacterium-contaminated feed (Doukas *et al.*, 1998).

Total Bacteria Count

Total bacterial count (TPC) of *A. hydrophila* in liver, kidney, spleen, intestine and muscle can be seen in Figure 2. Based on the results, total bacteria in organs of the infected fish with bath treatment of *A. hydrophila* and IM-injection yielded significantly

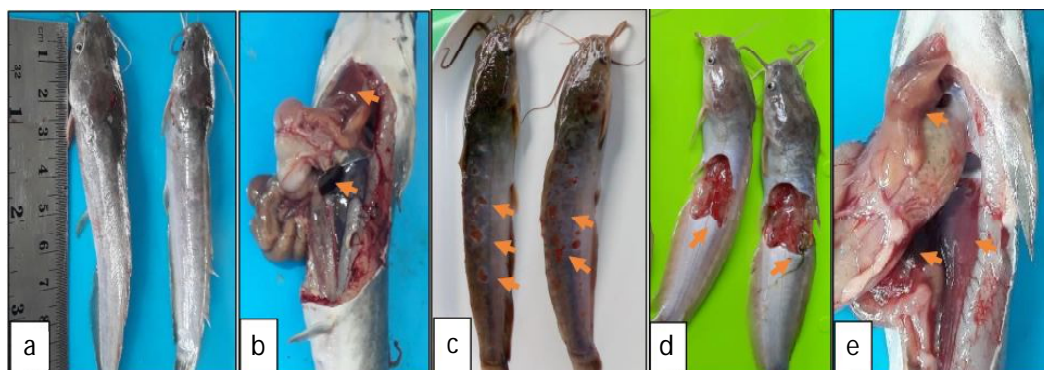
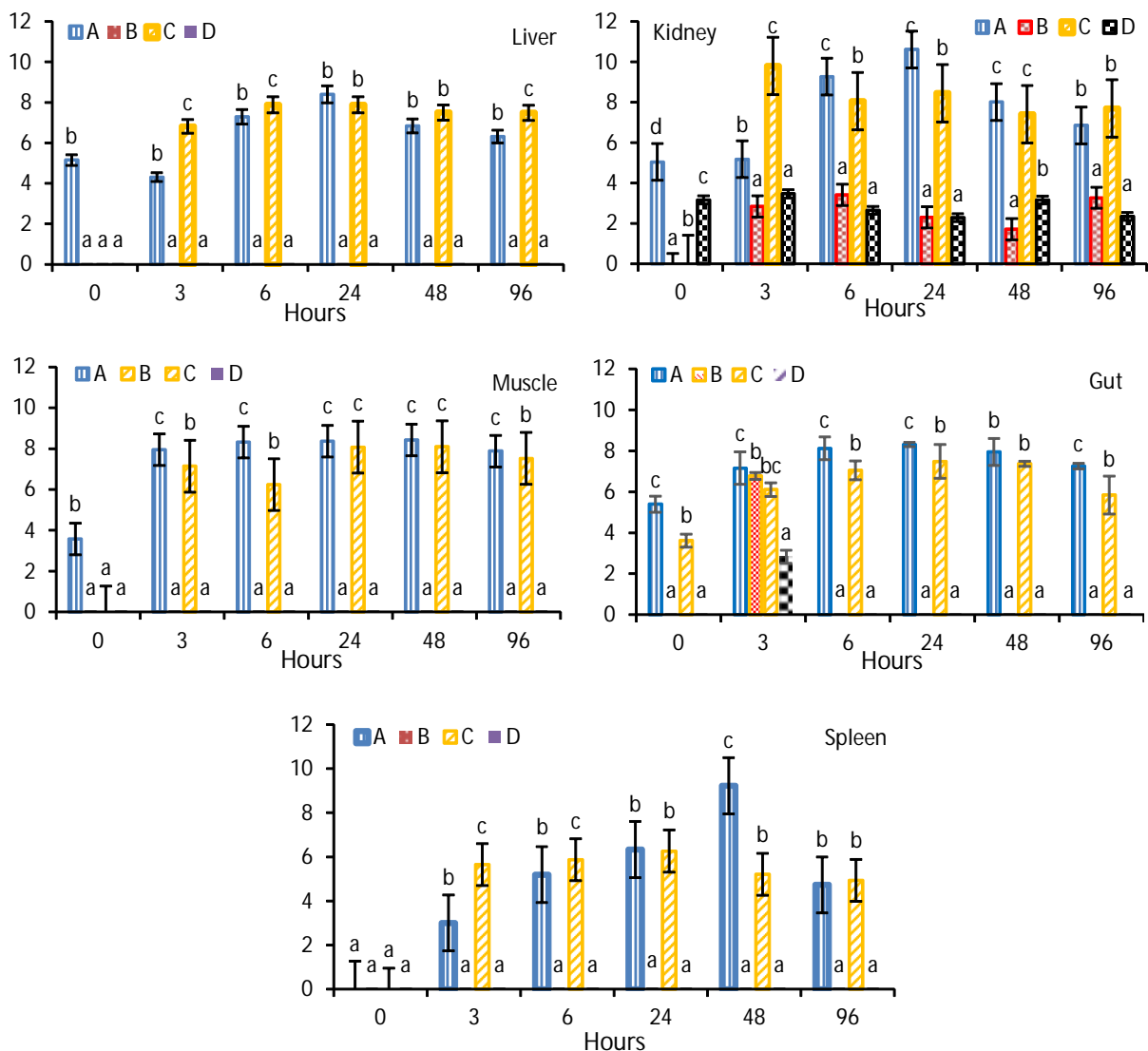


Figure 1. Healthy catfish (a); necropsy on healthy fish (b); fish muscular lesions in the immersion treatment with infection of *A. hydrophila* (c); fish muscular lesions in the IM-injection treatment (d); necropsy on diseased fish showing paler livers and kidneys and reddened intestines and spleens (e).

different bacterial counts than the bath treatment without *A. hydrophila* and PBS steril injection. There are no significant differences between of infected group of IM injection and bath infection. The highest bacterial count in the liver was seen in the bath treatment at 24 hpi at $8.4 \pm 0.13 \log_{10} \text{CFU mL}^{-1}$, from which point it went down at 48 hpi ($6.84 \pm 0.3 \log_{10} \text{CFU mL}^{-1}$) and further down at 96 hpi ($6.31 \pm 0.1 \log_{10} \text{CFU mL}^{-1}$). *A. hydrophila* were seen increasing significantly at 6 hpi ($7.88 \pm 0.28 \log_{10} \text{CFU mL}^{-1}$) in group of IM-injection (Figure 2a). The highest bacterial count in kidney for bath treatment was seen at 12 hpi ($10.61 \pm 0.58 \log_{10} \text{CFU mL}^{-1}$). As for the *A. hydrophila* count in the kidney of the IM-injected fish, the highest was at 3 hpi ($9.79 \pm 0.72 \log_{10}$

CFU mL^{-1}) and the second highest was at 96 hpi ($7.69 \pm 0.76 \log_{10} \text{CFU mL}^{-1}$) (Figure 2b).

A. hydrophila in the muscle was found at 0 hpi in immersed group with infection. In challenge fish with IM-injection group, *A. hydrophila* were found at three through 96 hpi (Figure 2c). The *A. hydrophila* in the intestine was found the highest at 12 hpi in both group immersed with infection ($8.31 \pm 0.11 \log_{10} \text{CFU mL}^{-1}$) and the group of IM-injection ($7.48 \pm 0.82 \log_{10} \text{CFU mL}^{-1}$) (Figure 2d). Meanwhile in spleen, the highest *A. hydrophila* count was found at 48 hpi both in group of immersed with infection ($9.22 \pm 0.98 \log_{10} \text{CFU mL}^{-1}$) and in group of IM-injection active *A. hydrophila* isolate $6.26 \pm 0.49 \log_{10} \text{CFU}$



Note: Immersion with *A. hydrophila* infection (A); immersion without *A. hydrophila* infection (B); *A. hydrophila* IM-injection (C); PBS steril injection (D)

Figure 2. TPC assays of several organs (Different letters in the same pattern show a significant difference (P < 0.05)).

mL⁻¹ (Figure 2e). In group of immersed without infection and PBS steril injection *A. hydrophila* were not found.

The total bacterial count showed that *A. hydrophila* found in several organs in 0 hour. Yardimci & Aydin (2011) state that although *A. hydrophila* has been known to be a fish pathogen, this bacterium is also part of normal microflora in healthy fish, so its natural presence does not necessarily indicate disease.

Histology

The histological observation on the kidney, liver, spleen, muscle, and intestine of all infected group showed that pathological changes in the organs started at 6 hpi, marked with bleeding and inflammatory cell infiltration. Pathological changes in kidney at 24 hpi shows that congestion mostly in major blood vessels, and necrosis, which was marked with mononuclear inflammatory cell infiltration in the multifocal areas of the parenchyma. An aggregates of highly pigmented phagocytes (melanomacrophage centers or MMCs) in the parenchym that was filled with hemosiderin, lipofuscin, and ceroid appeared regularly at 48 hpi (Figure 3b). Pathological changes in the livers and spleen appeared at 24 hpi. In the liver, most

hepatocytes seemed degraded with a congestion of the major blood vessel in the parenchyma (Figure 3d), while in the spleen MMCs were seen regularly in the parenchyma (Figure 3f). Observation on the muscle revealed that there was mononuclear inflammatory cell infiltration in the multifocal areas of the epidermis (Figure 3h), while observation on the intestine revealed that there was mononuclear inflammatory cell infiltration in the multifocal areas of the mucosa (Figure 3j). In observation at 96 hpi, there were a lot of Gram-negative bacteria spread on the serosal surface of the body cavity, parenchyma, and blood vessels, especially in the kidney.

The parameter level of severity based on Grizzel & Kiryu (1993) showed that infection with bath treatment caused systemic aeromoniasis infection (score 3) commenced at 24 hpi in the liver, kidney, muscle, and intestine, but in spleen it started at 48 hpi as same as in IM-injection method. In the non-infected caudal-fin-clipped fish, the level of severity stood at score 3, with limited wound infection on the skin and the muscle at 24 hpi and 48 hpi. Histological findings on the uninfected fish did not any show pathological changes, with a score of 0 (Figure 4). Extensive necrotic changes related to bacterial colonies

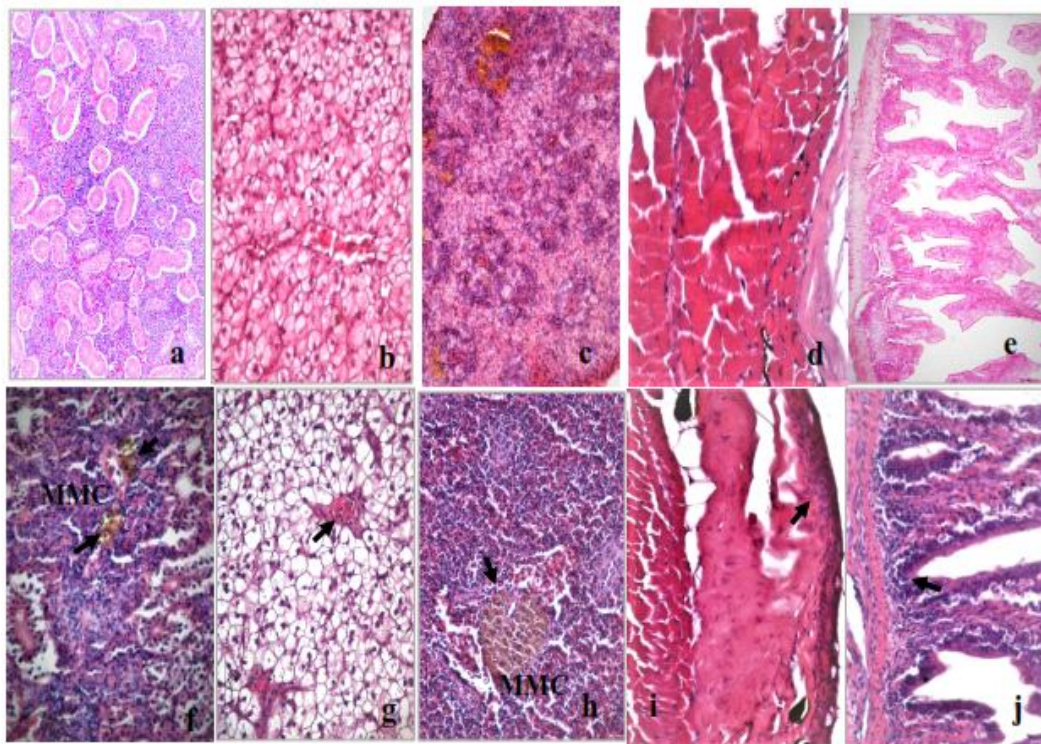
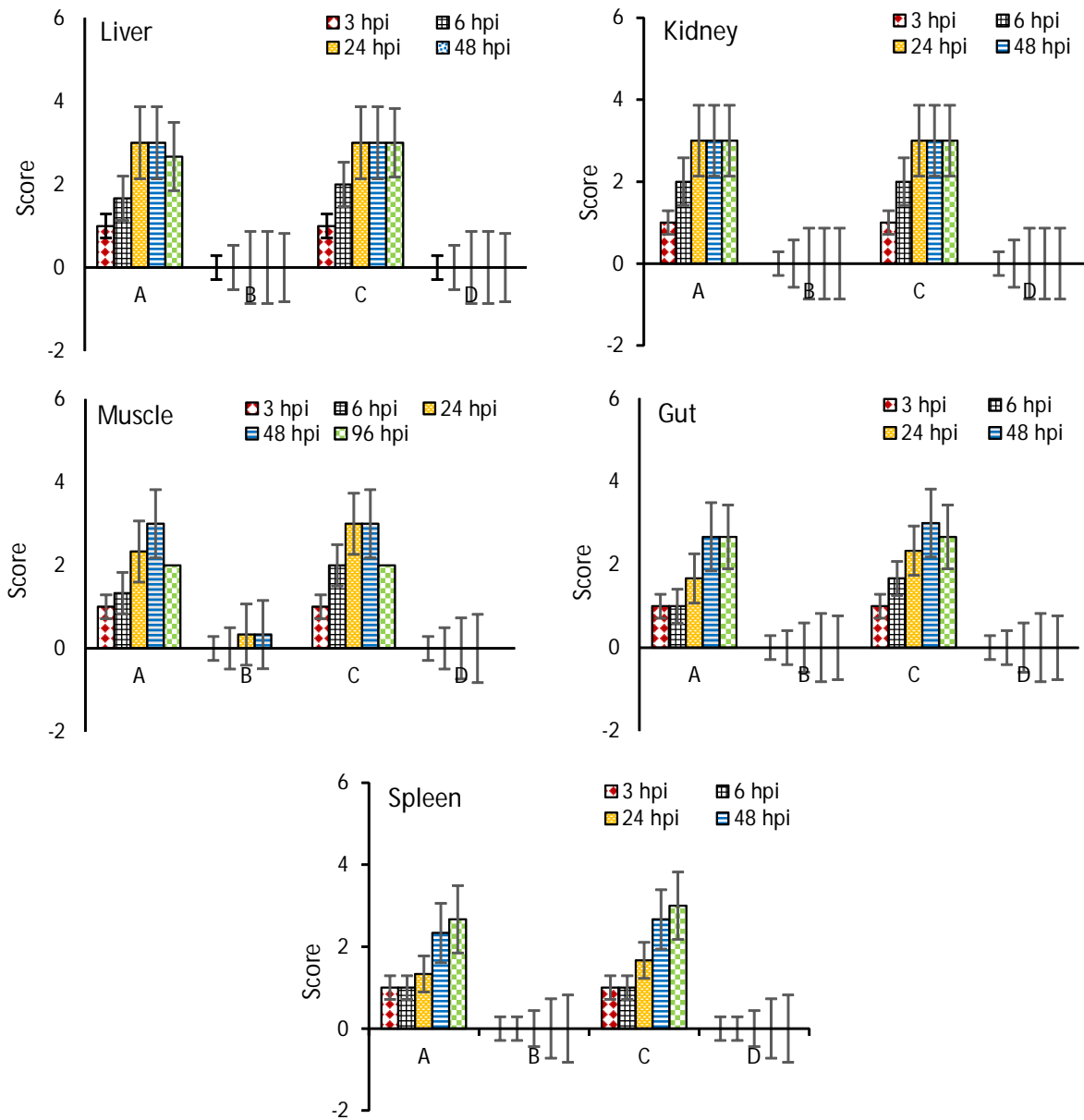


Figure 3. HE staining in several organs of experimental fish. Kidney of the uninfected fish (a); kidney of the infected fish (b); the liver of the uninfected fish (c); the liver of the infected fish (d); the spleen of the uninfected fish (e); the spleen of the infected fish (f); the muscle of the uninfected fish (g); the muscle of the infected fish (h); the intestine of the uninfected fish (i); the intestine of the infected fish (j); x400 magnification, bar: 20 μ m.



Note: Immersed with *A. hydrophila* infection (A); immersed without *A. hydrophila* infection (B); *A. hydrophila* IM-injection (C); PBS sterile injection (D)

Figure 4. Scoring of the severity level of *A. hydrophila* infection in the organs in each treatment.

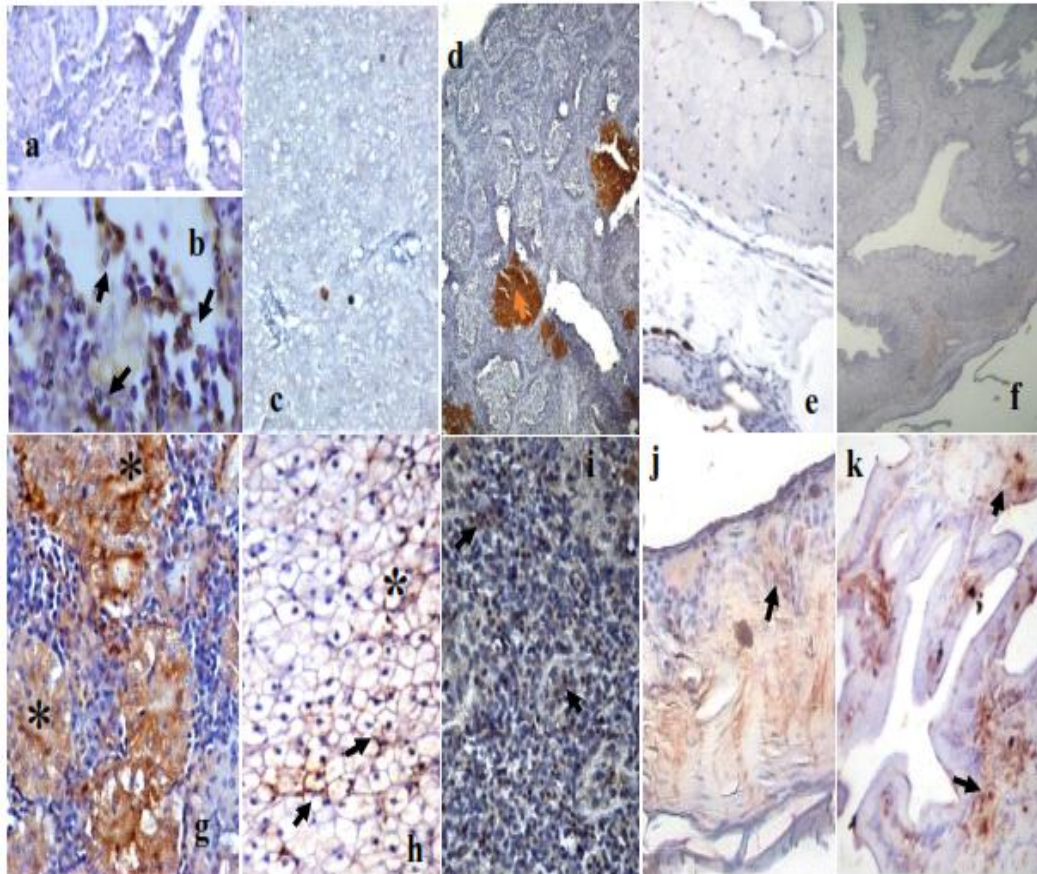
especially occurred in the kidney of the infected fish. Less extensively seen in the liver, spleen, intestine, and muscle. The uninfected group, no lesions were detected in the tissue.

TNF- α Visualisation and Distribution

TNF- α was detected all over the organ examined in immunohistochemical staining. TNF- α was detected in the cytoplasm, showing a morphology typical to monocytes/macrophages (Figure 5c), both in the infected group and in the uninfected group. TNF- α were seen secreted in the parenchyma tissue and intravascularly. Inflammatory cell infiltration as

leucocytes was seen abundant in the parenchyma tissue of the spleen and kidney. The difference in the number of TNF- α producing cells (expressed as mean \pm SD) between the two groups was significantly different in all samples, immunoreactive cells in the infected group compared with the uninfected group were observed at 6, 24, 48, and 96 hpi (Figure 6).

TNF- α was detected in the liver of the infected group at the 6 hpi which was significantly different from the uninfected group, and the highest production of TNF- α occurred at 24 hpi in the infected group with IM-injected (Figure 6a). In the kidney, the highest TNF production was detected at 24 hpi in



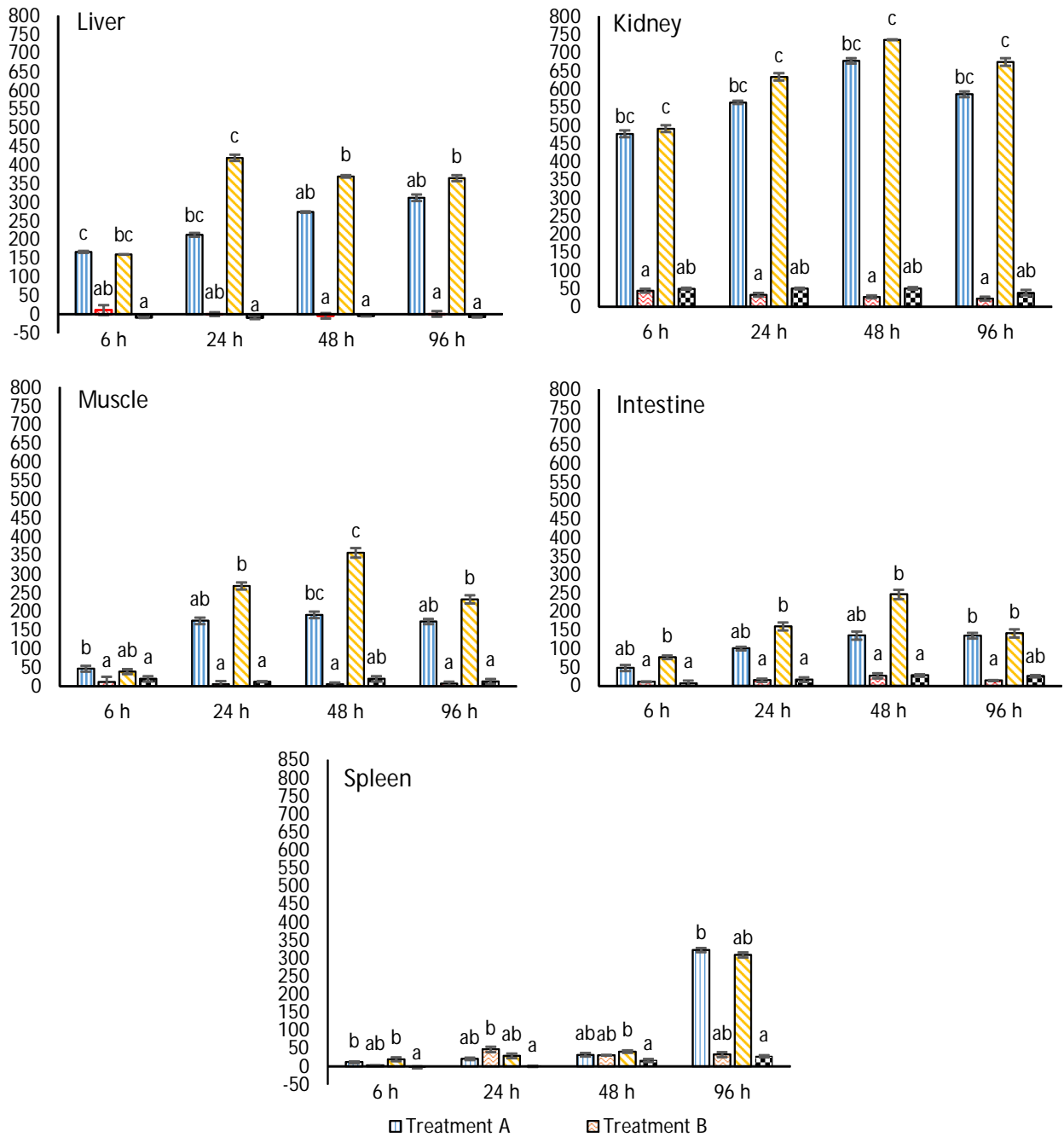
Note: Immunohistochemical (IHC) staining of TNF- α in several organs of experimental fish. Kidney of uninfected fish (a); TNF- α in infected kidney, TNF- α in the cytoplasm of monocytes (b); TNF- α in cytoplasm (c); liver of uninfected fish (d); TNF- α in infected liver (e); liver of uninfected fish (f); TNF- α in infected spleen (g); muscle of uninfected fish (h); TNF- α in muscle (i); intestine of uninfected fish (j); TNF- α in infected intestine (k); magnification 40x (scale bar: 50 μ m)

Figure 5. TNA- α immunohistochemical staining.

group by intramuscular injection which was not significantly different from the group infected by the immersed method but significantly different from the non-infected caudal-fin-clipped fish and the PBS sterile injection group (Figure 6b). The highest TNF- α concentration in the intestine was seen at 48 hpi in group of intramuscularly injected with active *A. hydrophila* isolate (Figure 6c). Similarly in the muscle, the highest TNF- α count was observed at 48 hpi in the IM-injected group (Figure 6d). The highest TNF- α concentration occurred at 96 hpi in spleen and showing a significant difference with uninfected group (Figure 6e). No significant difference was found at the sampling time in the uninfected group. Reactivity to TNF- α antigens in MMCs in the spleens at 96 hpi showed that there were lymphocyte-like cells.

As was observed in this research, the TNF- α kinetics after *A. hydrophila* infection at the liver, kidney, muscle, intestine, and spleen at some points in

time (Figure 6) was related to the level of lesion severity, and the increased number of *A. hydrophila* spread to each organ of the test fish within three hours after infection, followed by a change in the degree of pathological lesions. Progression into systematic *Aeromonas* infection took place around 24 hpi to 48 hpi. At the liver, *A. hydrophila* artificial infection caused systemic aeromoniasis infection and manifestation of fibrosis, necrosis, loss of hepatocyte wall, and hemosiderin deposit at the livers, in which case the hepatocytes experienced fairly severe degeneration. The histological observation by Al-Yahya *et al.* (2017) showed that the liver is an organ that suffers fairly severe necrosis, in which case the hepatocyte arranging at the liver shows some cells with vacuolation. An explanation for this, according to Afifi *et al.* (2000), is that the toxins and extracellular products produced by *A. hydrophila*, such as haemolysin, protease, and elastase, cause necro-



Note: Immersed and Ah infected (A); immersed, not Ah-infected (B); Ah IM-injected (C); PBS sterile injection (D)

Figure 6. TNF- α production in several organs.

sis at the liver. The highest bacterial count occurred at the liver at 24 hpi; at the same hour, the highest TNF- α count was also produced.

The two artificial infection models in this research caused systemic aeromoniasis infection in the kidney. The highest *A. hydrophila* count was found at 24 hpi, and at 48 hpi TNF- α production was the highest. At 96 hpi, the bacterial count decreased, and so did the TNF- α production. As a whole, the TNF- α production in the kidney was the highest among all the organs observed. Coscelli *et al.* (2016) say that,

the kidney is a hematopoietic organ, so it contains and produces a large number of macrophages that are capable of recognizing bacterial components and of mediating immune response (Rebl *et al.*, 2007) (Boehm *et al.*, 2012). Hwang *et al.* (2014) state that the kidney is an important source of TNF- α during the early phase of infection, and Rauta *et al.* (2012) and Press & Evensen (1999) say it is because the dominant phagocytic activity of the interstitial tissue. Falco *et al.* (2012) observed increased TNF- α gene expression in the leucocytes of the kidney of the common

carp (*Cyprinus carpio*) and the macrophages of kidney Atlantic salmon (*Salmo salar*) infected with *A. salmonicida* (Fast *et al.*, 2009). Observation of the kidney of the turbot that was infected with *A. salmonicida* showed that this organ suffered the most severe lesions in acute infection, which probably was related to a higher number of TNF- α than identified (Svendsen *et al.*, 1999).

The IM-injection method caused systemic *Aeromonas* infection at the muscle at 24 hpi, while the bath method did at 48 hpi. Although in the clinical symptom observation the muscles underwent visible lesions, the TNF- α production was lower than that of the livers and the kidneys despite the significant increase in the number since 6 hpi in comparison to the control. The abundant bacterial count since 6 hpi was probably because muscles were the first organs exposed to *A. hydrophila* due to artificial infection and the highest TNF- α production in the muscles peaked at 48 hpi. In the intestines, artificial infection by IM-injection caused systemic aeromoniasis infection, while infection by bath caused latent infection toward systemic aeromoniasis infection. As is seen in Figure 6, the intestines were the organs with the lowest TNF- α production among the organs observed. The highest TNF- α production and *A. hydrophila* count in the intestines appeared at 48 hpi.

TNF- α production was particularly rendered by activated macrophages and a number of antibacterial mechanisms involved in the innate immune system during acute bacterial infection (Zhu *et al.*, 2013; Ishibe *et al.*, 2009; Forlenza *et al.*, 2011). TNF- α -producing cells that demonstrated granulocyte morphological feature were also identified, particularly in the blood vessels. This finding is in accordance with the phagocytic function and capacity of immunocompetent cells. Coscelli *et al.* (2016) state that in the acute phase of *A. salmonicida* infection, monocyte/macrophage increase indicates their activation. The increase of the number of TNF- α -producing cells showed the increase of the number of immunoreactive cells in the experimental fish, indicating that the increase of TNF- α production was a response to bacterial infection (Coscelli *et al.*, 2016).

The TNF- α kinetics in spleens was seen different from that in other organs, in which case the bacterial count and level of lesion severity were not directly followed by TNF- α production, which only reached at 96 hpi. Spleen is a lymphoid organ (Abbas *et al.*, 2017), received macrophages from the kidneys, and the response would occur later with time (Coscelli *et al.*, 2016). Ronza *et al.* (2013) explain that the monocytic nature of melanomacrophages in spleen of te-

least fish is immunoreactive to TNF- α antigens so that it's showing melanomacrophages' ability to produce cytokines. In a study that used 10 turbot, only one expressed TNF- α in spleen MMCs at 96 hpi, suggesting that it probably takes strong antigenic stimulations for melanomacrophages to produce TNF- α . This shows its relationship with lymphocyte stimulation and proliferation as well as the commencement of adaptive responses (Bermúdez *et al.*, 2006). Such a finding proves that MMCs are involved in the innate immune response to bacterial infection. MMC is a structure that is responsible for the degradation, damage, or aging of the cells of an organism, erythrocytes, and endogenous materials such as haemosiderin, lipofuscin, and melanin (Agius *et al.*, 2003; Leknes, 2007).

In the present study, the two artificial infection models in the *C. gariepinus* induced an increase of TNF- α production in relation to the increase of bacterial count, followed by tissue destruction that marked the level of *A. hydrophila* infection at each organ. The increase of bacterial count was accompanied by the increase of TNF- α production, and vice versa, suggesting that TNF- α inhibited *A. hydrophila* growth, although this caused pathogenesis promotion. Given the lesion in the tissue and the relationship with sepsis pathogenesis, the increased TNF- α production is not to be overlooked, although it is not measured in a direct manner. Ronza *et al.* (2015) demonstrated the adverse effects of TNF- α production in the lesion development in the intestines of turbot that underwent enteromixosis. The rise of TNF- α production was also related to the tissue functional feature of the organ (Coscelli *et al.* 2016). To avoid severe tissue damage effects of *A. hydrophila* infection in catfish, advanced knowledge of the expression of other cytokines is deemed necessary in order to optimize the expression and activity of TNF- α , hence producing beneficial immune response induction.

CONCLUSION

Artificial infection by clipping the caudal fin of a *C. gariepinus* and immersing the fish in a medium with active *A. hydrophila* isolates was proven to cause systemic aeromoniasis infection in the liver, kidney, intestine, muscle, and spleen with clinical symptoms and pathological changes as those in artificial infection by intramuscular injection. Immunohistochemical staining of TNF- α antigens showed that *A. hydrophila* acute infection led to TNF- α production increase in infected fish. TNF- α detection indicated that immune response was induced efficiently in the lesions formed by *A. hydrophila* infection in catfish. A

higher rate of bacterial growth at the organ caused a higher rate of TNF- α production, and vice versa, showing the role of TNF- α in the reactivity of the immune system in *A. hydrophila* infection in catfish. The kinetics of TNF- α production in the kidney and spleen was different in the infected fish because the organ structure and function were different in responding to bacterial infection.

ACKNOWLEDGEMENTS

Acknowledgements are due to the Agency for Marine and Fisheries Research and Human Resources (BRSDMKP) of the Ministry of Marine Affairs and Fisheries (KKP), the Center for Freshwater Aquaculture Research and Fisheries Socialization (BRPBATPP) Bogor, The Fish Disease Control Research Installation (IRP2I) Depok, our fellows at IRP2I Depok, and the Primate Research Center of IPB University Bogor.

REFERENCES

- Abbas, A.K., Litchman, A.H., & Pillai, S. (2017). Cellular and molecular immunology. 9th ed., Elsevier.
- Adams, A. & de Mateo, M.M. (1994). Immunohistochemical detection of fish pathogens. In *Techniques in Fish Immunology 3* (J.S. Stolen, T.C. Fletcher, S.L. Kaattari & A.F. Rowley, eds), p. 133-144.
- Afifi, S.H., Al-Thobiati, S., & Hazaa, M.S. (2000). Bacteriological and histopathological studies on *Aeromonas hydrophila* infection of Nile tilapia (*Oreochromis niloticus*) from fish farms in Saudi Arabia. *Assiut. Vet. Med. J.*, 42, 195-205.
- Agius, C. & Roberts, R.J. (2003). Melano macrophage centres and their role in fish pathology. *Journal of Fish Diseases*, 26, 499-509.
- Al-Yahya, S.A., Ameen, F., Al-Niaem, K.S., Al-Sa'adi, B.A., & Mostafa, A.A. (2018). Histopathological studies of experimental *Aeromonas hydrophila* infection in blue tilapia, *Oreochromis aureus*. *Saudi Journal of Biological Sciences*, 25(1): 182-185. <https://doi.org/10.1016/j.sjbs.2017.10.019>.
- Assefa, A. & Abunna, F. (2018). Maintenance of fish health in aquaculture: Review of 342 epidemiological approaches for prevention and control of infectious disease of fish. *Vet. Med. International*, 343, 1-10. <https://doi.org/10.1155/2018/5432497>.
- Bermúdez, R., Vigliano, F., Marcaccini, A., Sitjà-Bobadilla, A., Quiroga, M., & Nieto, J. (2006). Response of Ig-positive cells to *Enteromyxumscophthalmi* (Myxozoa) experimental infection in turbot, *Scophthalmus maximus* (L.): A histopathological and immunohistochemical study. *Fish and Shellfish Immunology*, 21, 501-12.
- Boehm, T., Hess, I., & Swann, J.B. (2012). Evolution of lymphoid tissues. *Trends in Immunology*, 33, 315-21.
- Coscelli, G.A., Bermúdez, R., Losada, A.P., & Faílde, L.D. (2014). Acute *Aeromonas salmonicida* infection in turbot (*Scophthalmus maximus* L.) histopathological and immunohistochemical studies. *Aquaculture*, 430, 79-85.
- Coscelli, G., Bermúdez, R., Ronza, P., Losada, A.P., & Quiroga, M.I. (2016). Immunohistochemical study of inducible nitric oxide synthase and tumour necrosis factor alpha response in turbot (*Scophthalmus maximus*) experimentally infected with *Aeromonas salmonicida* subsp. *salmonicida*. *Fish and Shellfish Immunology*, doi: 10.1016/j.fsi.2016.07.022.
- Czarniecki, C.W. (1993). The role of tumor necrosis factor in viral disease. *Antiviral Res.*, 22, 223-58.
- Doukas, V., Athanassopoulou, F., Karagouni, E., & Dotsika, E. (1998). *Aeromonas hydrophila* infection in cultured sea bass, *Dicentrarchus labrax* L., and *Puntazzo puntazzo* Cuvier from the Aegean Sea. *Journal of Fish Diseases*, 21, 317- 320.
- Falco, A., Frost, P., Miest, J., Pionnier, N., Irnazarow, I., & Hoole, D. (2012). Reduced feed with β -glucan supplements. Inflammatory response to *Aeromonas salmonicida* infection in common carp (*Cyprinus carpio* L.). *Fish and Shellfish Immunology*, 32, 1051-7.
- Fast, M., Tse, B., Boyd, J., & Johnson, S. (2009). Mutations in the *Aeromonas salmonicida* subsp. *salmonicida* type III secretion system affect Atlantic salmon leucocyte activation and downstream immune responses. *Fish and Shellfish Immunology*, 27, 721-8.
- Forlenza, M., Fink, I.R., Raes, G., & Wiegertjes, G.F. (2011). Heterogeneity of macrophage activation in fish. *Developmental and Comparative Immunology*, 35, 1246-1255.
- Grizzle, J.M. & Kiryu, Y. (1993). Histopathology of gill, liver, and pancreas, and serum enzyme levels of channel catfish infected with *Aeromonas hydrophila* complex. *Journal of Aquatic Animal Health*, 5, 36-50.
- Hal, A.M. & El-Barbary, M.I. (2020). Gene expression and histopathological changes of Nile tilapia (*Oreochromis niloticus*) infected with *Aeromonas hydrophila* and *Pseudomonas fluorescens*. *Aquaculture*, 526. doi.org/10.1016/j.aquaculture.2020.735392.

- Hong, S., Li, R., Xu, Q., Secombes, C.J., & Wang, T. (2013). Two types of TNF- α exist in teleost fish: phylogeny, expression, and bioactivity analysis of type-II TNF- α in rainbow Trout *Oncorhynchus mykiss*. *The Journal of Immunology*, 191, 5959-72.
- Hwang, S.D., Shim, S.H., Kwon, M.-G., Chae, Y.S., Shim, W.J., Jung, J.-H., Kim, J.-W., Park, C.-I. (2014). Molecular cloning and expression analysis of two lipopolysaccharide-induced TNF- α factors (LITAFs) from rock bream, *Oplegnathus fasciatus*. *Fish and Shellfish Immunology*, 36, 467-474.
- Ishibe, K., Yamanishi, T., Wang, Y., Osatomi, K., Hara, K., Kanai, K., Yamaguchi, K., & Oda, T. (2009). Comparative analysis of the production of nitric oxide (NO) and tumor necrosis factor-alpha (TNF- α) from macrophages exposed to high virulent and low virulent strains of *Edwardsiella tarda*. *Fish and Shellfish Immunology*, 27, 386-9.
- Janda, J.M. & Abbott, S.L. (2010). Pathogenicity and infection: Taxonomy *Aeromonas* the genus. *Clin Microbiol Rev.*, 23(1), 35-73. doi: 10.1128/CMR.00039-09.
- Jiang, Q., Chen, W., Qin, Y., Huang, L., Xu, X., Zhao, L., & Yan, Q. (2017). AcuC, a histone deacetylase, contributes to the pathogenicity of *Aeromonas hydrophila*. *Microbiology*, 6(4), e00468. <https://doi.org/10.1002/mbo3.468>.
- Kadowaki, T., Harada, H., Sawada, Y., Kohchi, C., Soma, G.-I., & Takahashi, Y. (2009). Two types of tumor necrosis factor alpha in blue fin tuna (*Thunnus orientalis*) genes: Molecular cloning and expression profile in response to several immunological stimulants. *Fish and Shellfish Immunology*, 27, 585-94.
- Leknes, I.L. (2007). Melano-macrophage centres and endocytic cells in kidney and spleen of pearl gouramy and platyfish (Anabantidae, Poeciliidae: Teleostei). *Acta Histochemica*, 109, 164-8.
- Madigan, M.T., Martinko, J.M., & Parker, J. (2011). Brock biology of microorganisms. 13th ed. USA: Prentice-Hall Inc.
- Nya, E.J. & Austin, B. (2011). Development of immunity in rainbow trout (*Oncorhynchus mykiss*, Walbaum) to *Aeromonas hydrophila* after the dietary application of garlic. *Fish and Shellfish Immunology*, 30, 845-50.
- Press, C.McL. & Evensen, Ø. (1999). The morphology of the immune system in teleost fish. *Fish and Shellfish Immunology*, 9, 309-18.
- Rauta, P.R., Nayak, B., & Das, S. (2012). Immune system and immune responses in fish and their role in comparative immunity study: A model for higher organisms. *Immunology Letters*, 148, 23-33.
- Ronza, P., Bermúdez, R., Losada, A.P., Sitjà-Bobadilla, A., Pardo, B.G., & Quiroga, M.I. (2015). Immunohistochemical detection and gene expression of TNF- α in turbot (*Scophthalmus maximus*) enteromyxosis. *Fish and Shellfish Immunology*, 47, 368-76.
- Ronza, P., Losada, A.P., Coscelli, G.A., Faílde, L.D., Sancho, A.R., & de Azevedo, A.M. (2013). Expression of TNF- α in Teleost Fish: An Immunohistochemical Study. *Journal of Comparative Pathology*. 148:55.
- Reimchen, T.E. & Temple, N.F. (2004). Hydrodynamic and phylogenetic aspects of the adipose fin in fishes. *Can. J. Zool.*, 82, 910-916.
- Rebl, A., Siegl, E., Köllner, B., Fischer, U., & Seyfert, H.-M. (2007). Characterization of twin toll-like receptors from rainbow trout (*Oncorhynchus mykiss*): Evolutionary relationship and induced expression by *Aeromonas salmonicida salmonicida*. *Developmental and Comparative Immunology*, 31, 499-510.
- Roca, F.J., Mulero, I., López-Muñoz, A., Sepulcre, M.P., Renshaw, S.A., & Meseguer, J. (2008). Evolution of the inflammatory response in vertebrates: Fish TNF- α is a powerful activator of endothelial cells but hardly activates phagocytes. *The Journal of Immunology*, 181, 5071-81.
- Shoemaker, C.A., Mohammed, H., Bader, T.J., Peatman, E., & Beck, B.H. (2018). Bath vaccination with an inactivated virulent *Aeromonas hydrophila* bacterin protects hybrid catfish (*Ictalurus punctatus* x *Ictalurus furcatus*) from motile *Aeromonas septemimia*. *Fish and Shellfish Immunology*, 82, 239-242. doi: 10.1016/j.fsi.2018.08.040.
- Svendsen Y.S., Dalmo, R.A., & Bøgvold, J. (1999). Tissue localization of *Aeromonas salmonicida* in Atlantic salmon, *Salmo salar* L., following experimental challenge. *Journal of Fish Diseases*, 22, 125-31.
- Wellmer, A., Gerber, J., Ragheb, J., Zysk, G., Kunst, T., Smirnov, A., Bruck, W., Nau, R. (2001). Effect of deficiency of tumor necrosis factor alpha or both of its receptors on *Streptococcus pneumoniae* central nervous system infection and peritonitis. *Infect. and Immun.*, 69(11), 6881-6886. DOI: <https://doi.org/10.1128/IAI.69.11.6881-6886.2001>.

- Yambot, A.V. (1998). Isolation of *Aeromonas hydrophila* from *Oreochromis niloticus* during fish disease in Filiphina. *Asian Fisheries Science*, 10, 347-354.
- Yardimci, B. & Aydin, Y. (2011). Pathological findings of experimental *Aeromonas hydrophila* infection in nile tilapia (*Oreochromis niloticus*). *Ankara Üniv Vet Fak Derg.*, 58, 47-54.
- Zhang, D., Xu, D., & Shoemaker, C. (2016). Experimental induction of motile *Aeromonas septicemia* in channel catfish (*Ictalurus punctatus*) by waterborne challenge with virulent *Aeromonas hydrophila*. *Aquaculture Reports*, 3, 18-23.
- Zhang, A., Chen, D., Wei, H., Du, L., Zhao, T., & Wang, X. (2012). Functional characterization of TNF- α in grass carp anterior kidney leukocytes: Induction and involvement in the regulation of NF- κ B signaling. *Fish and Shellfish Immunology*, 33, 1123-32.
- Zhu, L.Y., Nie, L., Zhu, G., Xiang, L.X., Shao, J.Z. (2013). Advances in research of fish immune-relevant genes: A comparative overview of innate and adaptive immunity in teleosts. *Developmental and Comparative Immunology*, 39, 39-62.