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Histopathological changes in gold fish (*Carassius auratus* (Linnaeus, 1758)) infected by *Aeromonas hydrophila* bacteria with various densities

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ABSTRACT

The purpose of this study was to distinguish histopathological changes in carp fish (*Carassius auratus*) infected by *Aeromonas hydrophila* with different densities. Five treatments were used in the survey for this research method. The treatments used were bacterial infusion of *A. hydrophila* in the test fish through intramuscular injection with various densities, namely: A. without infection, B: 10^5 CFU/mL, C: 10^6 CFU/mL, D: 10^7 CFU/mL, and E: 10^8 CFU/mL. The parameters observed were histopathological changes in gill organs, kidneys and hearts of carp fish (gold fish). Data from histopathological observations were analyzed descriptively. The results present gill organs, liver and kidneys of carp fish infected with the bacteria *A. hydrophila* at various densities experiencing histopathological changes in the form of hemorrhages, necrosis and congestion and hyperplasia of the renal tubules. Other changes that occur were in the kidney organ experiencing a melanomacrophage center (MC). Tubules were coated with connective tissue and lymphocyte infiltration. The most severe organ damage that occurs in carp fish infected with the bacteria *A. hydrophila* at a density of 10^8 CFU/mL (treatment E) is shown by a low survival of 5%.

***Keyword*:** histopathology, clinical symptoms, *Aeromonas hydrophila*, *Carassius auratus*

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1. INTRODUCTION

Carp fish/gold fish (*Carassius auratus*) is one of the freshwater ornamental fish which is popular in all circles of society and has economic value that is quite important. Carp fish/gold fish cultivation activities cannot be separated from disease problems which can cause relatively large monetary losses. Pecuniary losses that occur due to disease attacks can cause a longer period of fish preservation, and can even cause mass death. Diseases in fish can be caused by various types of disease-carrying agents, one of which is due to bacteria pathogens.

The *Aeromonas hydrophila* bacterium is one of the pathogenic bacteria that can cause mass death in freshwater fish. As according to Anyanwu *et al.* (2015) *A. hydrophila* bacteria greatly influence freshwater fish farming and can cause epidemics of diseases with high mortality rates (80 - 100%) in a short period of time (1-2 weeks). The same thing was stated by Yin *et al.* (2010) that the infection of *A. hydrophila* bacteria can cause the death of aquaculture fish up to 80%.

The attack of *A. hydrophila* on fish can cause pathological changes such as acute, chronic and latent infections. Characteristic of *A. hydrophila* chronis infection is that fish can live for weeks, fish mortality occurs gradually and the cumulative mortality rate is high (Zhang 2016). According to Hardi *et al*. (2018) changes in organ tissue showed different damage at different levels of bacterial density. The higher the concentration of bacteria in the blood, the more damaged the organ will be, which can lead to non-functioning organs.

Histopathological observation of organs infected with *A. hydrophila* can help to determine the damage or abnormalities that occur in the tissue more clearly and precisely. Through histopathological observation, the level of tissue damage from infected organs will be generated. Histopathological observations can be employed to find out the symptoms of the disease early, because frequent attacks on fish do not show clinical symptoms or show the same clinical symptoms as other bacteria.

Information on disease attacks associated with histopathological organ damage in carp fish / gold fish is still lacking, so research on histopathological changes in fish organs infected with pathogens, such as *A. hydrophila* is needed, as a first step in the diagnosis of infectious diseases in fish.

The purpose of this study was to distinguish changes in histopathology of carp fish / gold fish infected with *A. hydrophila* bacteria at each density.

2. MATERIALS AND METHODS

The materials used in this study were gold fish (*Carassius auratus*) oranda strain as a test fish weighing 3-6 g / head as many as 100 fish originating from Depok Ornamental Fish Cultivation Center, isolates of *Aeromonas hydrophila*, xylol, ethanol absolute, ethanol 90%, etanol 80%, hematoxylin eosin (HE), aquades, tryptic soy agar (TSA), and tryptic soy broth (TSB).

The research method used was a survey with five treatments. The treatment is given to infect *Aeromonas hydrophila* bacteria in test fish by intramuscular injection method with different densities, namely A: without bacterial infection (control), B: 10^5 CFU/mL, C: 10^6 CFU/mL, D: 10^7 CFU/mL, and E: 10^8 CFU/mL.

3. RESEARCH PROCEDURE

The 60×40×35 cm^3 aquarium was cleaned, the inner part was soaked with Potassium Permanganate ($KMnO_4$) 5 ppm for 24 hours, then rinsed and dried. In aquaria, a lot of water and aerators, blowers, and water quality checkers are installed. Each aquarium was filled with 20 test fish, then acclimatized for 14 days. During acclimatization the test fish was given commercial feed of the HI-PRO-VITE brand with a protein content of 30% carried out by adlibitum. After acclimatization of the test fish infected with *A. hydrophila* bacteria with different densities, i.e. 10^5 CFU/mL, 10^6 CFU/mL, 10^7 CFU/mL, and 10^8 CFU/mL through injection method as much as 0.1 mL intramuscularly, *A. hydrophila* bacteria were used as purification products in TSA media. Different bacterial densities are obtained by diluting one bacterial ose serially in 5 mL TSB media.

After injection, the test fish was maintained for 4 days and observed by organ tissue histopathologically. Necropsy for target organ retrieval of the test fish (gills, liver and kidney) was performed on days 1, 3, 5, 7, and 14, after infection. Necropsy results of each target organ were made histopathological preparations. The first stage in making histopathological preparations, namely fixation, by means of target organ tissue was to soak in Bouins solution for 48 hours. The second stage of the target organs that have been fixed was to dehydrate by soaking in 70% alcohol solution for 48 hours; then the soaking was continued in a row with alcohol 80%, 90%, 95% for 2 hours each, and 100% alcohol for 12 hours. The third stage was clearing by means of target organs soaked with alcohol-xylol in a ratio of 1 : 1 for 30 minutes, attended by soaking in xylol three times, each of 30 minutes. Soaking the target organ in xylol-paraffin (1 : 1) for 45 minutes was the fourth stage of infiltration. The fourth stage is embedding, which is to go into paraffin into the cell, by organ tissue soaking in paraffin three times, each of 45 minutes. The fifth stage is blocking, which is printing the network so that it is easy to turn off. The sixth stage of network sectioning uses a microtome with 4 micrometers thickness of 3 slices, each slice being inserted into 50% alcohol and distilled water at 40 ºC, then arranged on the object glass. The seventh stage is carried out by tissue staining, beginning with hydration, which is removing paraffin by soaking in xylol solution 2 times, each for 3 minutes, then soaking in 100% alcohol solution 2 times, each for 3 minutes, followed by alcohol 95, 90, 80, 70, and 50%, 3 minutes each and then washed with distilled water 2 times. The eighth stage was stained by breaking down the tissue in the solution of hematoxylin for 10 minutes, then washing with running water for 10 minutes, followed by immersion in the eosin solution for 3 minutes, then washing with distilled water. The last stage of colouring is to do dehydration by soaking the staining samples in 50% alcohol, 2 times each, followed by alcohol 70, 85, 90, and 100% each, for 2 minutes and then with xylol 2 times, each for 3 minutes. Then they are covered with a cover glass that has been dripped with mounting, dried in an oven at 40 ºC for 24 hours, and then observed under a microscope.

4. RESULTS AND DISCUSSION

4. 1. Histopathology of gills

Based on the results of histopathological observations, staining HE (Hematoxylin Eosin) on gill of gold fish infected with *A. hydrophila* bacteria showed hyperplasia, hemorrhage, congestion and necrosis (Table 1).

Table 1. Damage to the gill tissue of gold fish infected with Bacteria *A. hydrophila*

Treatment	Gill tissue day-to-day observations				
	1	**3**	**5**	**7**	**14**
A (control) B (10^5 CFU/mL)	- HP	- HP	- HP	- HP	- HP
C (10^6 CFU/mL)	H, HP, N	HP	HP, K	HP	HP
D (10^7 CFU/mL)	HP	H, HP	HP, K	HP	HP
E (10^8 CFU/mL)	HP	HP			

Information:

- : No damage occurred
H: Hemoraghi
HP: Hiperplasia
N: Necrosis
K: Congestion

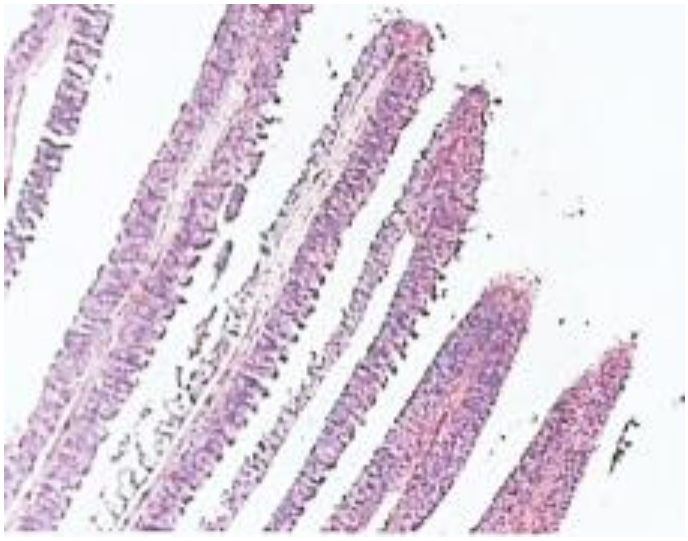

Figure 1. Histopathology of goldfish gills before being infected with *A. hydrophila* bacteria (A treatment / control)

Table 1 shows that the test fish that were not infected by *A. hydrophila* bacteria (treatment A) did not experience changes / gill tissue damage (Figure 1). Test fish infected with *A.*

hydrophila bacteria at all densities, starting from day 1 observations, had undergone gill tissue changes. Gill tissue in fish treatment test B (10^5 CFU / mL) from day 1 to day 14 presented experienced mild hyperplasia (Figure 2). Hyperplasia occurs due to an increase in the number of lamellar epithelial cells, causing the lamella to stick together and blend with one another, so that between the primary and secondary lamellae it cannot be seen and the gill lamellae appear larger than at normal conditions.

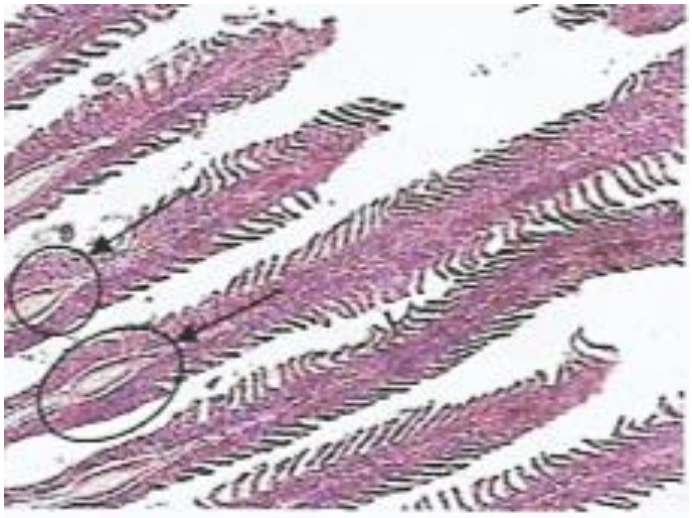

Figure 2. Hyperplasia of gills in carp / gold fish infected with *A. hydrophila* bacteria (400× magnification)

Fish gill tissue test on treatment C (10^6 CFU/ mL) on day 1, besides having hyperplasia, also experience hemorrhage (Figure 3) and necrosis (Figure 4).

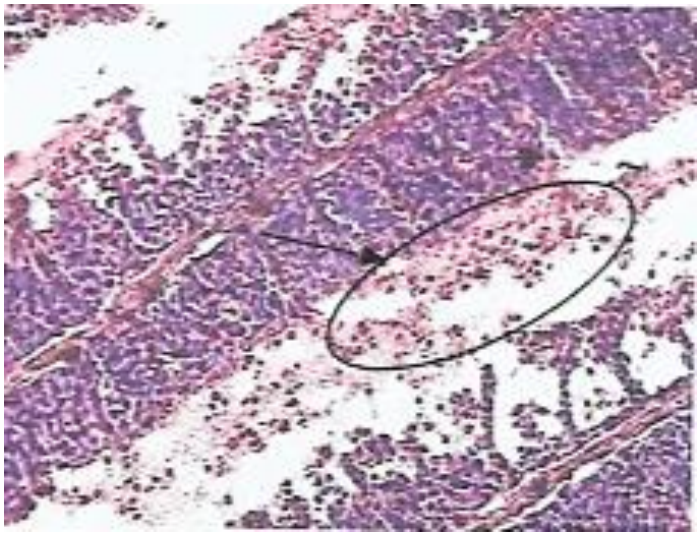

Figure 3. Hemorrhage in the gills of carp / gold fish infected with *A. hydrophila* (400× magnification)

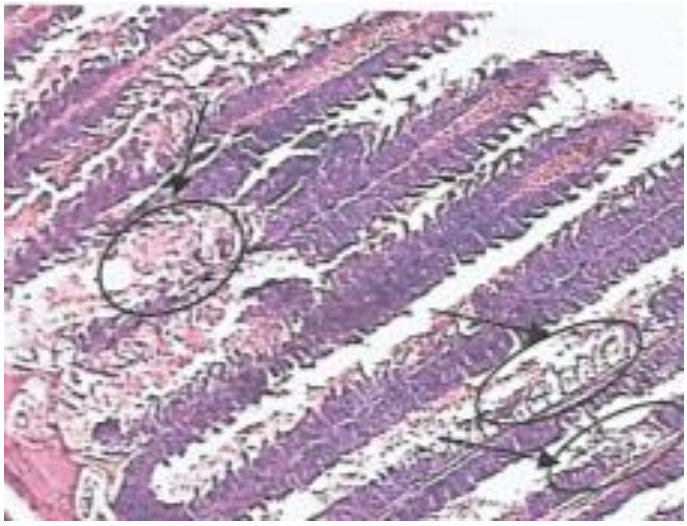

Figure 4. Necrosis of gill carp infected by *A. hydrophila* (400× magnification).

Hemorrhage is one of the circulation disorders where blood comes out of the blood vessels both out of the body of the fish and out of the body tissue of the fish. Necrosis is cell death or the end result of a degenerative change that cannot return to its original state. According to Burr *et al.* (2005) necrosis is caused by enzymatic degradation produced by *A. hydrophila*. Test fish that experienced hemorrhage and necrosis on the 3rd day of observation had experienced death, so the test fish that survived until the end of the observation (day 14) were the test fish that had hyperplasia.

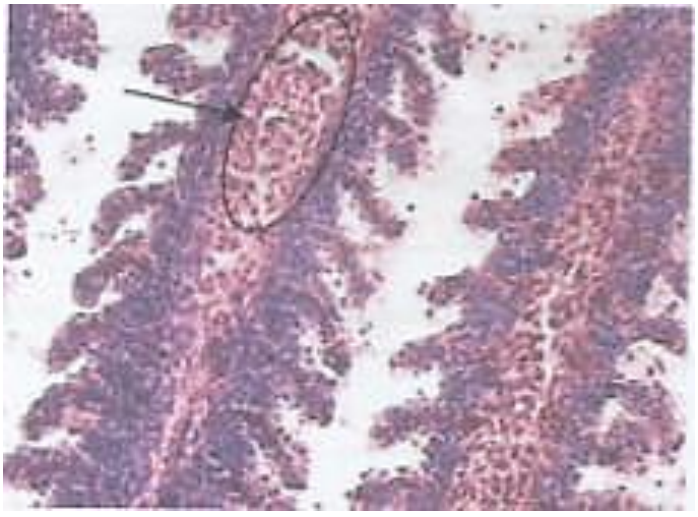

Figure 5. Congestion on the gills of carp / gold fish infected with *A. hydrophila* (400× magnification)

On observation day 5, apart from hyperplasia, congestion was also found (Figure 5). Congestion is the occurrence of blood containment caused by a disruption in circulation which

causes the fish to experience a lack of oxygen and nutrients. Congestion looks like cell swelling. This is due to the increase in cell size, due to the accumulation of fluid in the cell so that the cell looks enlarged. This disorder causes narrowed sinusoids and interrupted blood flow.

During the test fish gills in treatment D (10^7 CFU/mL), in addition to experiencing hyperplasia, also hemorrhage occurred on the 3rd day of observation. On the 5th day of observations, a congestion occurred. All test fish in treatment E (10^8 CFU/mL), from the start of observation on day 1, experienced the most severe hyperplasia compared to other treatments, so that at the end of the observation (day 14) the test fish survived only 5% with the condition of the gills that were broken. Damaged gill conditions can cause damaged red blood cells, and hemoglobin contained in red blood cells can not function to bind oxygen; as a result, the fish will lack of oxygen and the fish's body can not metabolize and eventually the fish will experience death.

4. 2. Histopathology of the Liver

Based on the results of histopathological observations of the kidney organs, several different damages were found in each treatment, namely necrosis, hemorrhage and congestion (Table 2).

Table 2. Damage to the liver tissue Carp fish / gold fish infected with bacterium *A. hydrophila*

Treatment	Liver tissue day-to-day observations				
	1	**3**	**5**	**7**	**14**
A (control)	-	-	-	-	-
B (10^5 CFU/mL)	H, K, N	K, N	N	N	K, N
C (10^6 CFU/mL)	N	H, N	N	N	N
D (10^7 CFU/mL)	N	N	K, N	K, N	K, N
E (10^8 CFU/mL)	K, N	N			

Information:

- : no damage — N: necrosis
H: hemoraghi — K: congesti

Histopathological observations of carp fish / gold fish treatment A (control) did not show any damage (Figure 6). After being injected with bacteria *A. Hydrophila*, the liver undergoes necrosis (Figure 7) which occurs every hour of observation in all treatments. The part of the

liver cell that experiences necrosis, looks damaged, stretched and died, while the part of the liver cell that does not experience necrosis is inflamed. According to Burr *et al.* (2005), necrosis is a degeneration caused by damage to the enzyme composition of cells, malnutrition, and glycogen depletion. Besides, necrosis in liver cells can also be caused by chronic anoxia.

The reasons for the vacuolation and necrosis of the liver were reported to be associated with toxins and extracellular products such as haemolysin, proteases, and elastases produced by *A. hydrophila* (Afifi *et al*., 2000, Laith & Najiah 2013).

The protease activity has an important role in the pathogenesis of *A. hydrophila* due to the ability of tissue damage and infection establishment by overcoming the host defense (Tomás 2012).

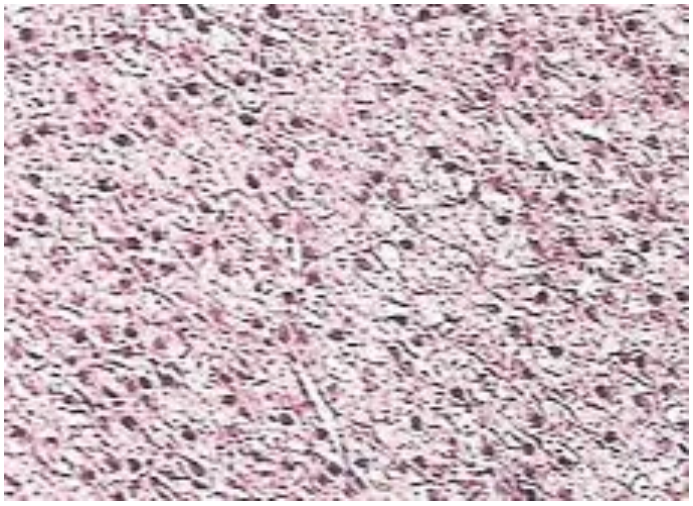

Figure 6. Histopathology of liver carp fish / gold fish before being infected with the bacterium *A. hydrophila* (A treatment / control)

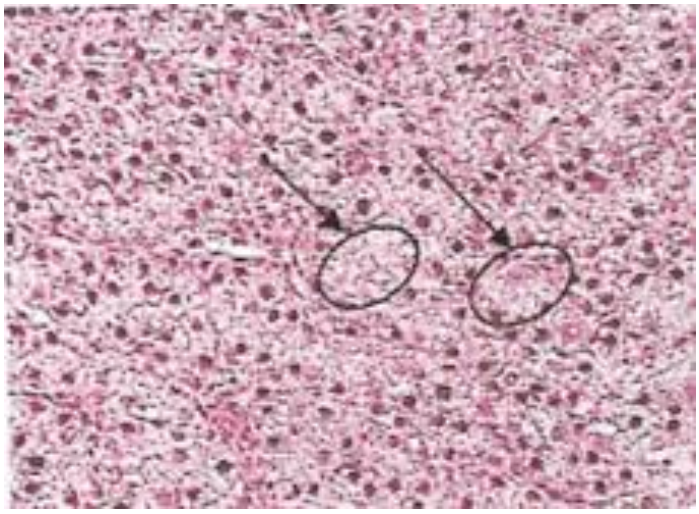

Figure 7. Histopathology of liver carp fish / gold fish infected with *A. hydrophila* (400×)

In addition to necrosis, the liver also experiences congestion and hemorrhage (Figure 8, and Figure 9). The presence of congestion and hemorrhage is seen on observations of days 1, 3, and 14, in treatment B, observations of day 3 in treatment C, treatment D is seen in observations of days 5, 7, and 14, and treatment E on day l. Congestion occurs due to the entry of toxic substances released by the bacteria *A. hydrophila* in the body of the fish that attack the liver. Liver cell congestion begins with swelling of cells, where liver cells enlarge and cause sinusoids to narrow so that blood flow is disrupted. This results in the holding of blood in several places.

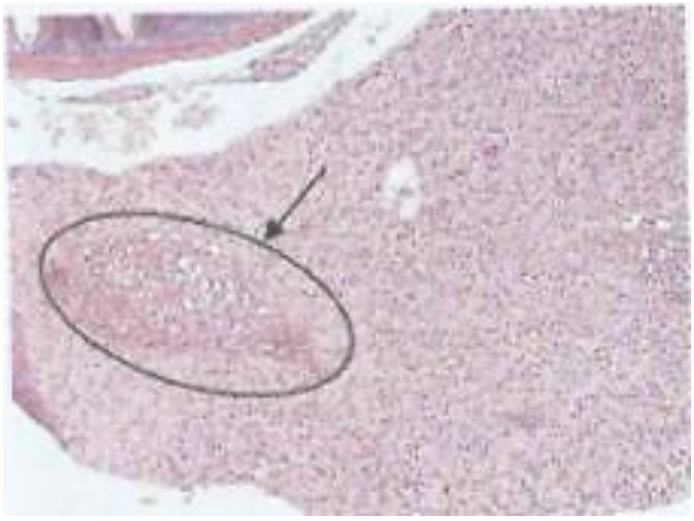

Figure 8. Congestion in liver carp fish / gold fish infected with *A. hydrophila* (100×)

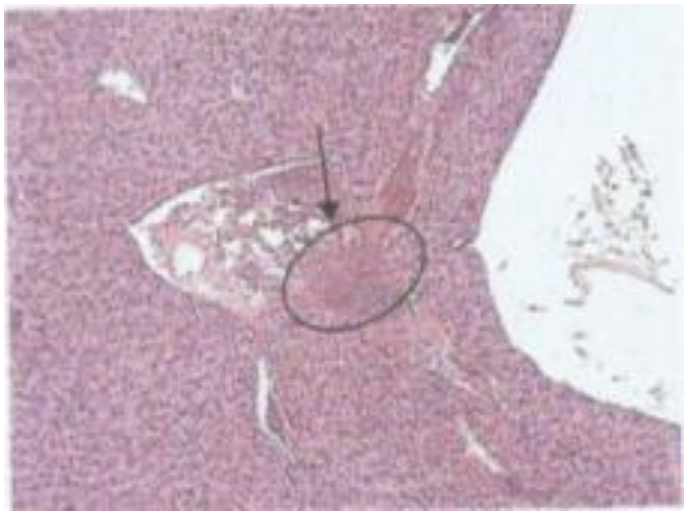

Figure 9. Hemorrhagic in liver carp Fish / gold fish infected with *A. hydrophila* (100×)

The liver is one of the organs in fish that is most often damaged (Stratev 2015, Roy *et al*. 2018). The liver receives 89% of the blood supply from the portal vein which flows into the

blood from the gastrointestinal system. The substance of the toxic and chemical substances absorbed into the portal vein is transformed to the liver. In addition, the liver also produces enzymes that act as biotransformations in various kinds of exogenous and endogenous substances that are eliminated by the body (Steinei and Boinick 2017). The liver functions in the digestive system, produces bile for detoxification, hematopoiesis (formation of red blood cells) and destroys red blood that is not used (Upadhyay 2017). According to Mounika *et al*. (2017) the liver functions in detoxification, synthesis of several components of blood plasma, storage of glucose in the form of glycogen and releasing glucose which is used as energy for fish.

4. 3. Kidney histopathology

Based on the results of histopathological observations of the kidney organs, several different damages can be found in each treatment, namely necrosis, hemorrhage, hyperplasia, Melanomacrophage Center (MMC), protein deposits, lymphocyte infiltration, and congestion (Table 3).

Table 3. Damage to Kidney Carp Fish / gold fish infected with *A. hydrophila* bacteria.

Treatment	Liver tissue day-to-day observations				
	1	**3**	**5**	**7**	**14**
A (control)	-	-	-	-	-
B (10^5 CFU/mL)	H, HP, N	N	MMC	H, K	HP
C (10^6 CFU/mL)	K	JI	HP, K	N	K, HP
D (10^7CFU/mL)	H, N	K	K, N	H, IL	K, N
E (10^8 CFU/mL)	HP	K			

Information:

H:	Hemoraghi	N:	Nekrosis
HP:	Hiperplasia	K:	Congestion
MMC:	Melanomacrofage Centre	JI:	Connective tissue
IL:	Lymphocyte Infiltration	- :	normal Kidney

Based on Table 3, kidney tissue damage varies with each treatment. In treatment A (control), there was no damage caused by the bacterium *A. hydrophila* but an abnormality was seen, namely protein deposition suspected to be a congenital abnormality (Figure 10).

Damage in the form of hemorrhage (Figure 11) occurred at treatment B-1 day. Other damages that were also found during the same observation and treatment period were hyperplasia of the kidney tubules (Figure 12) and necrosis (Figure 13).

Test fish in treatment D also resulted in damage in the form of hemorrhage on day 1 and day 7; besides, it was also found in treatment B on day 7. Renal tubular hyperplasia was found on the observation day 1. On treatment B on the 14th day, and treatment C on the 5th and 14th day, renal tubular hyperplasia was found.

Necrosis was also found in treatment D on observations of day 1, day 5 and day 14. Necrosis also occurred in treatment B on day 3 of observations, and treatment C on day 7 of observations.

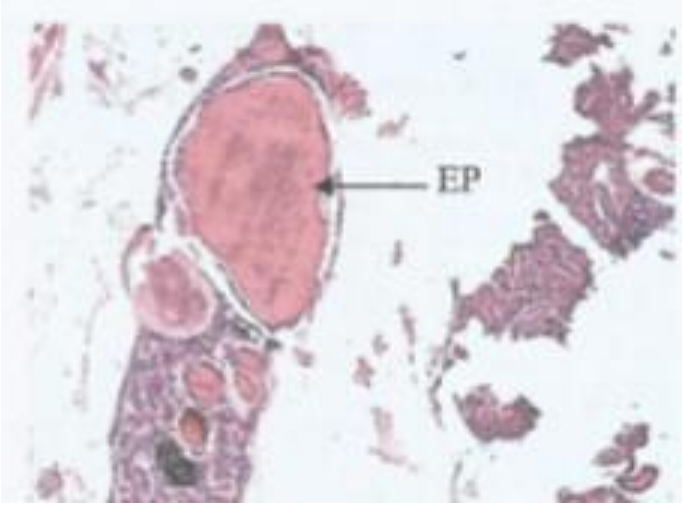

Figure 10. Histopathology of kidney carp fish / gold fish before being infected with *A. hydrophila* bacteria (Treatment A / Control).

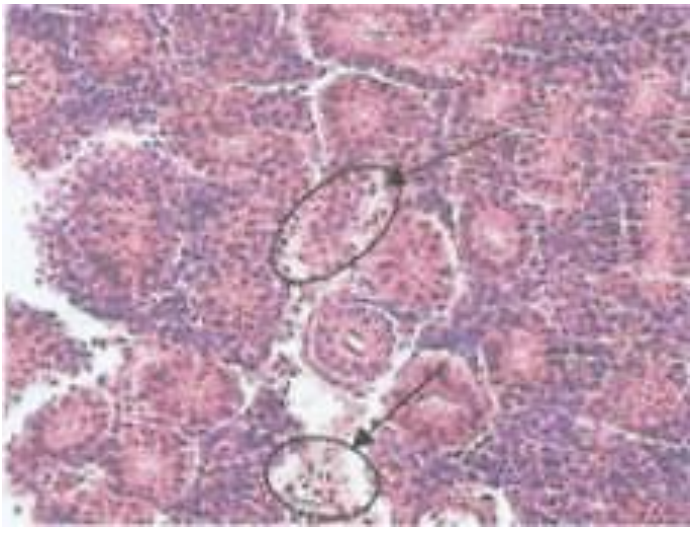

Figure 11. Hemorrhage in kidney carp fish / gold fish infected with *A. hydrophila* (400×).

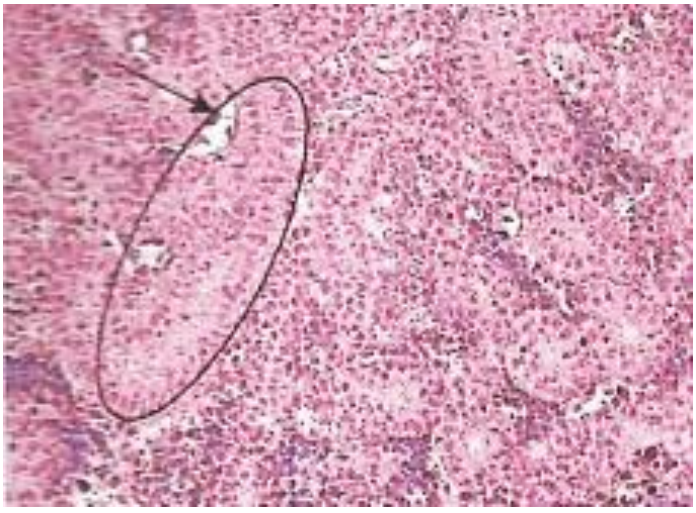

Figure 12. Hyperplasia in kidney tubules carp fish / gold fish infected with *A. hydrophila* (400×) bacteria.

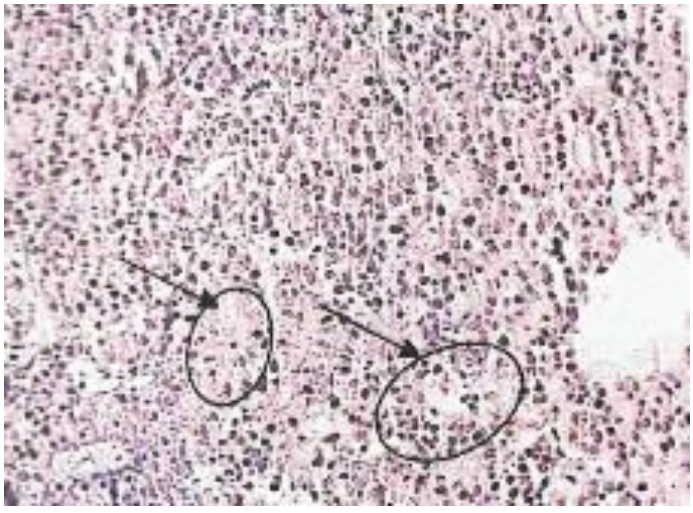

Figure 13. Necrosis of kidney tubules carp fish / gold fish infected with *A. hydrophila* (400×).

As the results of research conducted by Roy *et al.* (2018) on Nile tilapia *Oreochromis niloticus* kidney damage, degenerative changes occur with severe hemorrhages and extensive necrosis excretory tubules and glomeruli cells. The lumina of tubules that contained degenerated tubular epithelial cells with hyaline droplets accumulation and infiltration of lymphocytic cells (round cells with large blue staining nuclei) were clearly visible.

Other scrapes found in kidney carp fish / gold fish infected with *A. hydrophila* bacteria are congestion (Figure 14) and MMC (hemosiderin) (Figure 15). According to Ersa (2008), melanomakrofag center is a collection of macrophages that contain hemosiderin, lipofuschin and seroid as well as the melanin pigment that is found in most lymphoid tissues of teleost caused by inflammation. Melanomacrophage centers (MMC) are the collection of macrophages

that contain hemosiderin, lipofuchsin and ceroids as well as the melanin pigment. MMC is found in lymphoid tissue, especially teleostei fish caused by inflammation (Azad *et al.* 2001). In the present study, kidney, spleen and liver tissues have the presence of hemosiderin which proliferated into MMC. According to Couillard *et al.* (1999), MMC may contain four types of brown pigments known as melanin, lipofuscin, ceroid and hemosiderin. Baumgartner (2017) reported earlier that an acute septicemia will target liver and kidneys.

Lymphocyte infiltration (Figure 16) in the kidney occurs due to fish's resistance to the bacterial infection of *A. hydrophila* automatically. The function of lymphocytes is to function as the body's defense system, the way it works is by phagocyting foreign objects that enter the body. In addition, there are other abnormalities, namely kidney tubules which are covered in connective tissue (Figure 17).

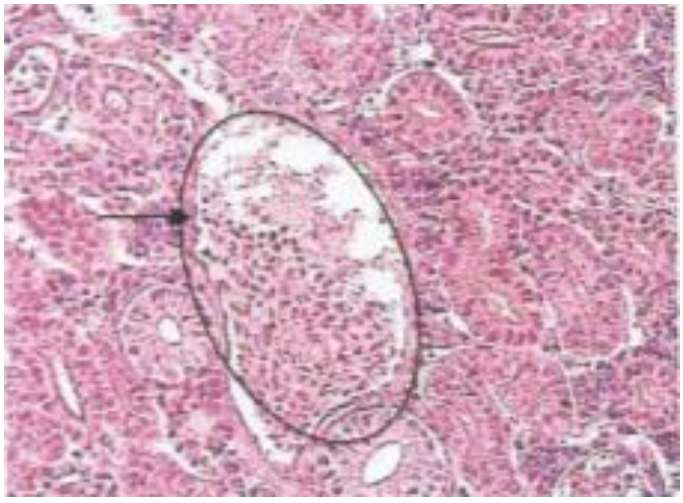

Figure 14. Congestion in kidney tubules carp fish / gold fish infected with *A. hydrophila* (400×).

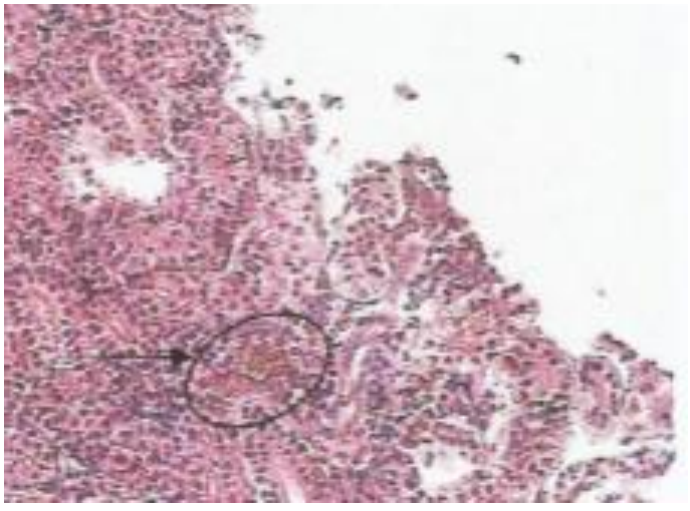

Figure 15. MMC kidney carp fish / gold fish infected with *A. hydrophila* (400×)

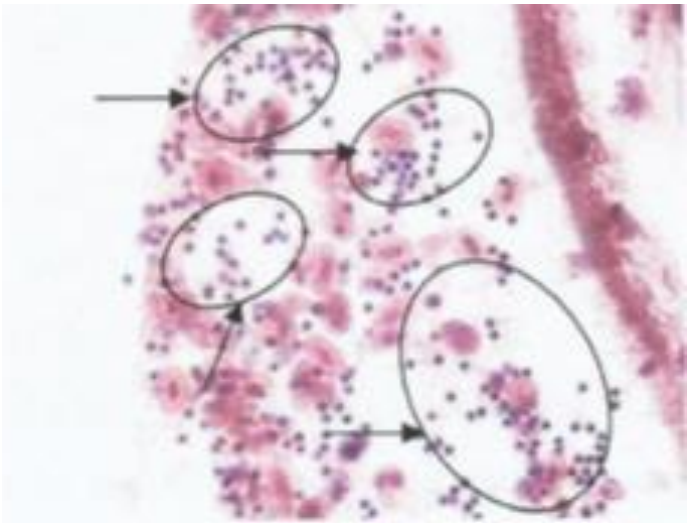

Figure 16. Lymphocyte infiltration in kidney carp fish / gold fish infected with *A. hydrophila* (400×).

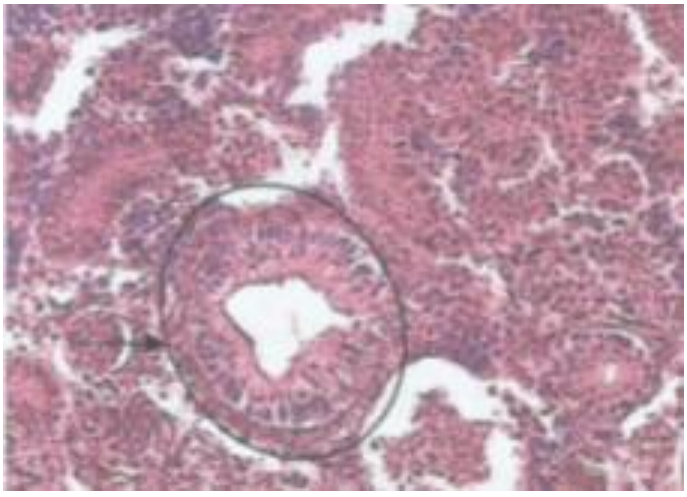

Figure 17. Kidney tubules coated in connective tissue in carp fish / gold fish infected with *A. hydrophila* (400×)

The hyaline droplets which were found in kidney were likely constituted with protein reabsorbed from the glomerular filtrate after being contaminated by bacterial toxin (Jiraungkoorskul *et al.* 2002).

Moderate aggregation of lymphocytic infiltration was also observed in kidney, liver and spleen (Stratev 2015). Lymphocytes are recognised as immuno-competent cells that commonly relate to immune response of the fish towards infection (Magnadottir 2010, Wang *et al*.2016).

4. 4. Level of Organ Damage

From the analysis of the gills, liver and kidney carp fish / gold fish infected with *Aeromonas hydrophila* bacteria during the study, it can be seen that each treatment has a different level of organ tissue damage (Table 4).

Table 4. Damage Rate of Organ Carp / gold fish infected with *Aeromonas hydrophila* bacteria.

Observed organs	Days to-	Treatment			
		B	**C**	**D**	**E**
Gill	1	HP	H, HP, N	HP	HP
	3	HP	HP	H, HP	HP
	5	HP	HP, K	HP, K	
	7	HP	HP	HP	
	14	HP	HP	HP	
	Damage rate	+	+	++	++
Liver	1	H, K, N	N	N	K, N
	3	K, N	H, N	N	N
	5	N	N	K, N	
	7	N	N	K, N	
	14	K, N	N	K, N	
	Damage rate	+	++	+++	+++
Kidney	1	H, HP, N	K	H, N	HP
	3	N	JI	K	K
	5	MMC	HP, K	K, N	
	7	H, K	N	H, L	
	14	HP	K, HP	K, N	
	Damage rate	+	+	+++	+++

Information:

HP	=	Hiperplasia	MMC	=	*Melanomacrophage Centre*
IL	=	Lymphocyte Infiltration	+	=	Light
H	=	Hemoraghi	++	=	Is
N	=	Nekrosis	+++	=	Weight
K	=	Congestion			
JI	=	Fish connective tissue			

Table 4 shows that organ damage in treatments B and C is mild to moderate damage, whereas organ damage in treatments D and E takes moderate to severe damage. Organ damage is said to be mild if there are only a few points of damage to the organ. Organ damage is said to be severe if the damage spreads (there are several points of damage) to the organ, whereas damage is moderate if the level of damage is between mild and severe. From the results of the analysis of the level of organ damage carp / gold fish infected with *A. hydrophila* bacteria, the most severe organ damage occurred in the treatment E (10^8 CFU / mL), but the observation of the level of organ damage in the treatment E was only carried out until the day 3. This is because the test fish in treatment E experienced a very high mortality that is equal to 95%, so that the next day (days 5, 7, and 14) can not take samples for histopathological examination.

From the description above it shows that the higher density of bacteria that is infected into the body of the fish results in more severe damage to the organ tissue. This is consistent with the statement of Hardi *et al.* (2018) that the higher the density of *Aeromonas hydrophila* bacteria will cause more severe damage to the fish's body

4. 5. Survival rate

Based on the observations of mortality of carp / gold fish (Appendix 7) during the infection period (14 days), the survival rate of carp fish / gold fish for each treatment (A, B, C, D, and E) varies (Figure 18).

The survival data of carp fish / gold fish show that the lowest survival rate was in treatment E. The low survival rate in treatment E was caused by bacterial infection *A. hydrophila* with the highest bacterial density of 10^8 CFU / mL which caused the fish to be infected, decreased immune system and impaired metabolism.

Carp Fish / gold fish in treatment A (control) and B (10^5 CFU / mL) showed a higher survival rate, because in treatment A (control) there was no injection of the bacterium *A. hydrophila*, whereas in treatment B, the density of the bacteria injected is the lowest one. This shows that the higher the density of the injected bacteria, the lower the survival rate. Carp Fish / gold fish infected with the bacterium *A. hydrophila* that can survive during the study are due to innate immunity from the fish itself. This is in accordance with the statement of Das *et al*. (2011) that fish infected with *A. hydrophila* that survive have high serum titers from antibodies such as IgM.

The mechanism of *A. hydrophila* in killing fish is by producing endotoxins and exotoxins which include hemolisin and proteases (Al-Fatlawy and Al-Ammar 2013, Silva *et al*. 2012, Doan 2013). Hemolisin is an enzyme that is able to lyse red blood cells and free up hemoglobin, whereas protease is a proteolytic enzyme that functions to fight the host's body's defense for

disease development and take stock of host nutrients to breed (Janda and Abbott 2010, Rozi *et al.* 2017, EL Malek 2017). Based on the description above, it shows that the density of bacteria infected with carp fish / gold fish is inversely proportional to the survival rate. The higher density of bacteria injected provides a lower survival rate.

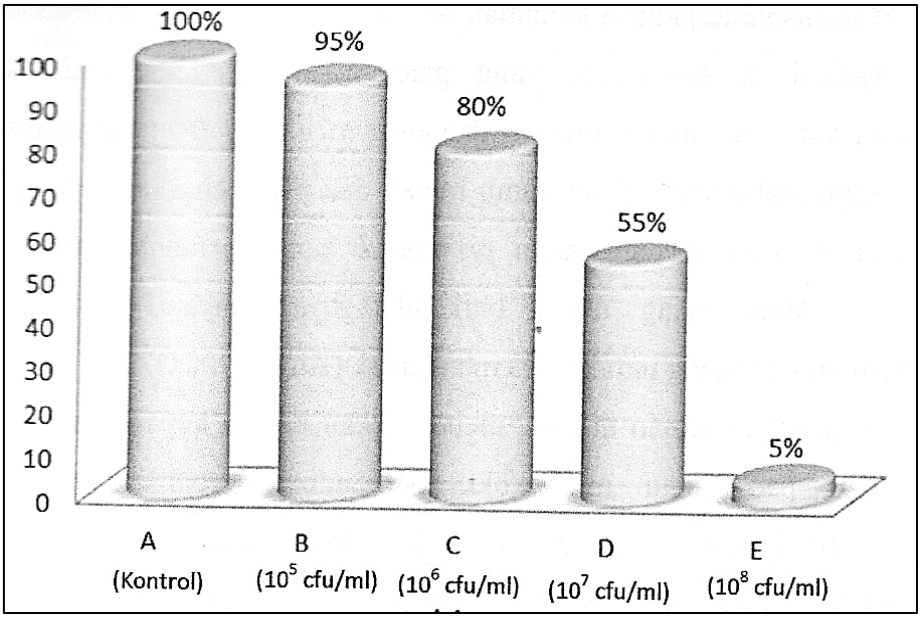

Figure 18. Survival rates of carp / gold fish infected with *A. hydrophila*

5. CONCLUSION

Carp Fish / gold fish infected with *Aeromonas hydrophila* bacteria at all densities undergo changes / damage to the tissues of the organs of the gills, liver and kidneys. Bacterial density of 10^8 CFU / mL showed the most severe damage and the lowest survival (5%).

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