



Virulence test of *Aeromonas hydrophila* bacteria on goldfish (*Carassius auratus*)

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Abstract

This research aims to determine the virulence of *A. hydrophila* bacteria with different densities which were infected to the goldfish (*Carassius auratus*). This research were used survey method with five treatments. The animal test were goldfish (*Carassius auratus*) infected by *Aeromonas hydrophila* bacteria with different density. The treatments divided into uninfected (Control / Treatment A), density of 10^5 cfu / ml (treatment B), density of 10^6 cfu / ml (treatment C), density of 10^7 cfu / ml (treatment D) and density of 10^8 cfu / ml (treatment E). The parameters observed were clinical symptoms which included fish damage body surface, feeding response, response to shock and survival rate. The data obtained were analyzed descriptively. The results of the study showed that the goldfish infected by *Aeromonas hydrophila* bacteria with different densities showed the different responses of surface damage such as inflammation, hyperemia/hemorrhage, ulcers, gill conditions, abscesses, response to feeding, and survival rate. *Aeromonas hydrophila* bacteria with a density of 10^8 cfu / ml (E treatment) resulted the most severe body damage, low response to food, and very low survival rate (5%). The conclusion of this research is high density infection of *Aeromonas hydrophila* bacteria on the goldfish will give the high risk severe surface damage and high mortality rate. *Aeromonas hydrophila* with a density of 10^8 cfu / ml (treatment E) infected in goldfish is categorized as a virulent.

Keywords: *Aeromonas hydrophila*, goldfish, virulent

1. Introduction

Bacterial diseases in fish especially caused by *Aeromonas hydrophila* can cause mass mortality in freshwater fish. Lukistowyati and Kumiasih (2012) ^[9] stated that *A. hydrophila* bacteria could greatly affected the freshwater fish farming and caused disease outbreaks with high mortality rate (80 - 100%) within a short period of time (1-2 weeks). Yin *et al.* (2010) ^[18] also stated that bacterial infection of *A. hidrophila* caused the death up to 80%. Based on several reports, these bacteria attacked and caused disease outbreaks in various types of freshwater fish both fish consumption and ornamental fish, including catfish (*Clarias sp.*) and tilapia (*Oreochromis niloticus*) (Anyanwu *et al.* 2015, El Deen *et al.* 2014) ^[1, 4],

Goldfish (*Carassius auratus*) is one of the freshwater fish which has an economic value and being popular as an ornamental fish. The activity of goldfish culture can not be separated from the health problems. One research conducted in Pekanbaru, showed that *A. hydrophila* in goldfish is the highest bacteria persentasenya compared with other types of pathogenic bacteria (Desriana and Prayitno 1999) ^[3]. *A. hydrophila* bacteria including pathogenic bacteria, virulence of pathogenic bacteria is determined by the amount or density of the bacteria. According to Huicab-Pech *et al.* (2016) ^[6] if the number of *A. hydrophila* in the waters exceeds 10^4 cells per milliliter can then be categorized as virulent bacteria. According to Yulianto *et al.* (2013) ^[19], changes in clinical symptoms and tissue damage depending on the level of bacterial density. The higher the density of bacteria in the blood, the more severe the organ damage is

marked by the absence of organ function, which can be observed with clinical symptoms such as loss of balance and damage to the external organs. The purpose of this study was to determine the most virulent bacterial density of *A. hydrophila* in the goldfish.

2. Materials and Methods

The ingredients used in this study were carp fish (*Carassius auratus*) Oranda strain with average initial body weight of 3-6 g from 100 fish, commercial feed with a protein content of 30% were given in *ad libitum*. At the first time, *Aeromonas hydrophila* were used and to be grown on the bacteria medium test Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) as general mediums, then *Aeromonas* Medium Base (RYAN) was selected as the medium to see the bacterial growth.

Survey method was used as a proper method in this research to test the *Aeromonas hydrophila* infection through intramuscular injection with five different bacteria density treatments, as follow:

A: without bacterial infection (control)

B: density of 10^5 cfu / ml

C: density of 10^6 cfu / ml

D: density of 10^7 cfu / ml

E: density of 10^8 cfu / ml

Five aquarium with the size of 60x40x35 cm³ were used to test the intramuscular infection treatments of *Aeromonas hydrophila*. After being prepared with hoses and blowers, sterilization was conducted to prevent the infection of others

bacteria. Fish were put into the aquarium with a density of 20 fish/aquarium, then being acclimatized within 14 days. Meanwhile, *Aeromonas hydrophila* suspension were grown in TSA medium, then incubated in incubator for 24 hours at 30°C. The growing rate was analyzed after 24 hours incubation period, then the characteristics of *Aeromonas hydrophila* were observed such as gram equation, morphological test, catalase test, oxidase test, and biochemical test. After that, *Aeromonas hydrophila* were re-incubated for 24 hours at a temperature of 30°C in TSA medium. One strip of *A. hydrophila* was dissolved in 5 ml TSB, then re-incubated for 24 h in an incubator at 30°C. After incubation period, the bacterial dilution was performed by using the aseptic reaction and micropipette tubes to obtain a suitable bacterial density of 10⁵ CFU /ml, 10⁶ CFU /ml, 10⁷ CFU /ml and 10⁸ CFU /ml. The test fish were infected by bacteria through intramuscular injection method of 0.1 ml. After being injected, the test fish were kept for 14 days and clinical symptoms were observed, such

as surface damage, response to feed, response to shock and survival rate. Observation of clinical symptoms and survival rate were recorded daily and analyzed descriptively. An easy way to comply with the paper formatting requirements is to use this document as a template and simply type your text into it.

3. Results and Discussion

3.1 Damage to the surface of the body

Observation of surface damage started from the beginning of injection until the end of observation (day 14), the data were recorded hourly. Fish body damage parameters consisted of inflammation, red spots to bleeding, ulcers, ulcers, changes of gill color and abscesses (out of the yellow liquid from the fish's belly) were observed. Based on the observations of infected fish with different density of *A. hydrophila* bacteria showed a different body damage (Table 1).

Table 1: Damage of Fish Body Surface after Injecting with Bacteria *A. hydrophila*

Day-	Treatments				
	A (Control)	B (10 ⁵ cfu/ml)	C (10 ⁶ cfu/ml)	D (10 ⁷ cfu/ml)	E (10 ⁸ cfu/ml)
1					
Hour-1	-	-	-	-	-
Hour-3	-	-	-	-	-
Hour-6	-	-	-	I	I
Hour-24	-	I	I	I, H, S	I, H, S
2	-	I	I	U, IP	U, PG, AB
3	-	I, H	I, H	PG, U	R, H
4	-	I, H	I, S	I, H	Dead
5	-	I	I	I	Dead
6	-	I	I	I	Dead
7	-	I	I	I	Dead
8	-	I	I	I	Dead
9	-	I	I	I	Dead
10	-	I	I	I	Dead
11	-	I	I	I	Dead
12	-	I	I	I	Dead
13	-	I	I	I	Dead
14	-	I	I	I	Dead

*Table indicated the damage of surface body from day 1 to day 14. In addition there was an extra observation in day 1 in every 1 hour, 3 hours, 6 hours, and 24 hours after injection. The capital letters in each column indicated: I = Inflammation; U = Ulcer; AB = Abscess; S = sore; PG = Pale gills; - = No damage; H = Hyperemia (Hemoraghi).

Damage were not observed on body surface of goldfish in treatment A (control) and fish showed a healthy and robustness until the end of the observation (Figure 1), it happened because fish in treatment A were not infected by *A. hydrophila* bacteria. On the 1st day, from hour-1 until hour-6 after infection, the fish on treatments B and C have not shown body damage, fish on D and E treatments have shown inflammatory body damage (Figure 2A). Significant body damage became clear on 24 hours after infection (Figure 2B).

Beside inflammatory occurred, also hyperemia / hemorrhagic and ulcers also found in treatment D and E 24 hours after infection (Figure 3), inflammation widened and appeared to the other side of the body. According to Li *et al.* (2013), the emergence of hemoraghi or red spots due to toxins released by *A. hydrophila* bacteria could spread throughout the body through the bloodstream and caused hemolysis and rupture of blood vessels.



Fig 1: Goldfish without infection by *A. hydrophila* bacteria (Treatment A)



Fig 2: Inflammation of goldfish after injection with *A. hydrophila* bacteria on day 1 at hour-6 (A) and hour-24 (B)

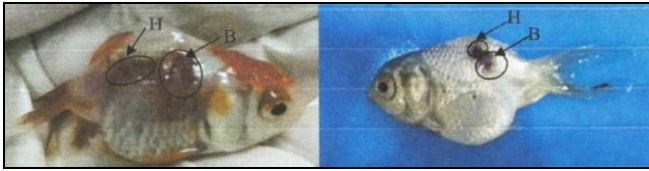


Fig 3: Hyperemia (H) and ulcers (U) in the goldfish after injection with *A. hydrophila* bacteria

On day 2, fish on treatment B and C only showed the body damage in the form of inflammatory, whereas the goldfish in treatment D and E were damaged in the form of ulcers (gaping wound) and gill pale (Figure 4), the most severe body damage occurred in treatment E (Fig. 4A) compared with the treatment D (4B), then showed the abscess (Figure 5).

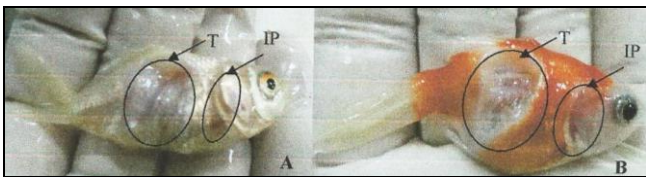


Fig 4: Ulcer and Gill Pale on a goldfish after injection by *A. hydrophila* Goldfish in treatment E in day 2 (A); Goldfish in treatment D in day 2 (B)

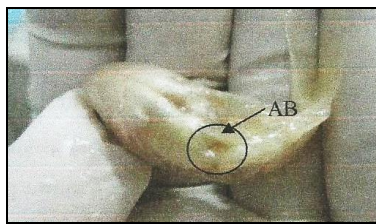


Fig 5: Abscess on the stomach of goldfish infected by *A. hydrophila*

On day 3, Treatment B and C showed inflammatory and hyperemia / hemoraghi, while the treatment D experienced more severe such as; ulcers, gills, pallor, inflammation and hemorrhagic, and the remain fish in treatment E were severed more damage inflammatory and hemorrhagic. According to Yardimci & Aydin (2011) [17] symptoms of inflammation and hyperemia is a clinical manifestation of *A. hydrophila*, skin redness or hyperemia is the first clinical sign of infection. *A. hydrophila* could recognized and fixed into the receptor cells and break down the host cells by producing extracellular enzymes such as hemolysin, proteases and elastase causing inflammation and developing into ulcers. Anyanwu *et al.* (2015) [1] also stated that hyperemia is an early response to pathogenic microbial infections, followed by inflammation, necrosis and ulcer formation. This hyperemia occurs due to erythrocyte mobility to the tissues where pathogens develop.

On day 4, the observation in treatment B and C showed suffering almost on the same part damage; inflammation and hemorrhage and ulcers. Inflammation was continued until the end of observation (day 14). The remaining test fish on treatment D suffered body damage in the form of pale ulcer and gill and inflammation continued until end of observation with more severe condition compared with treatment B and C. Moreover, the test fish on treatment E on day 4 experienced 95% mortality and the remaining fish (5%) severed bad condition in its wound.

According to Rozi *et al.* (2017), diseases caused by *A. hydrophila* bacteria show characteristics of body surface injuries, haemorrhages, especially in the gills, ulcers and flatulence. *A. hydrophila* bacteria can produce exoprotease enzymes. Such exo-protease enzymes that can cause the pathogenic of *A. hydrophila* because they can destruct or degrade connective tissue and muscle tissue such as collagen, elastin, gelatin, and so on. Experts conclude that exo-protease enzymes are a major virulence factor among other extracellular products secreted by bacteria. According to Sartika (2011) [16], *A. hydrophila* enzymes can cause damage to the infected body surfaces because of muscle and blood vessels have many protein contents.

From the above description shows the higher the density of bacteria infected in the body fish will lead to more severe body fish damage.

3.2 Feeding response

Based on the results of observations of fish response to the feed given, showed different responses for each observation time and each treatment (Table 2).

Table 2: Feeding response of goldfish after injection with bacteria *A. hydrophila*

Day-	Feeding response				
	A (Control)	B (10 ⁵ cfu/ml)	C (10 ⁶ cfu/ml)	D (10 ⁷ cfu/ml)	E (10 ⁸ cfu/ml)
1	++	-	-	-	-
2	++	-	-	-	-
3	++	-	-	-	-
4	++	+	+	-	Dead
5	++	+	+	+	Dead
6	++	++	++	+	Dead
7	++	++	++	+	Dead
8	++	++	++	++	Dead
9	++	++	++	++	Dead
10	++	++	++	++	Dead
11	++	++	++	++	Dead
12	++	++	++	++	dead
13	++	++	++	++	dead
14	++	++	++	++	dead

*Feeding response observed during the experimental period indicated by No Response (-), Small Response (+), and Normal Feeding Response (++)

In Table 2, on day 1 until day 3 all goldfish in treatment B, C, D and E were no showed any response to the feed, while in treatment A, response to the feed showed high and normal rate until the end of experiment. The absence of feeding response on day 1 showed that fish experienced post-injection stress with *A. hydrophila* bacteria. This result in line with the statement Hamid *et al.* (2016) [5] which stated that the clinical symptoms that caused the change in fish behavior such as swimming on the surface of water, decreased appetite as a result of fish experiencing stress.

According to Irianto (2005) [7] stress is a condition when animals are unable to regulate normal physiological conditions due to various adverse factors that affect their health. The absence of feed responses on observations on day 2 and 3 was due to the bacteria *A. hydrophila* already infected the goldfish so that the fish decreased the response to the feed given. Olga (2014) [12] stated that the decreasing of feeding response in fish is one of the clinical symptoms of *A. hydrophila* infected the body fish. Roy *et al.* (2018) [14]

stated that one of the target organs of attack of *A. hydrophila* is the kidney, where the disruption of these organs can affect the body's metabolic processes.

Observation on day 4 and 5 after the injection showed that the goldfish on treatment B and C started to show a response to the feed given, but the response shown was still lacking, as shown by the residual feed that accumulated in the bottom of the waters. This indicated that the given feed were not consumed in its entirety. Treatment D showed that fish were not responded the feed given until day 4. On the day 5, the test fish started to show response to but it was still lacking and lasting until day 7. Meanwhile in treatment E, fish test most (95%) have experienced death and alive fish condition were already severe.

The goldfish infected with *A. hydrophila* bacteria 10^5 CFU / ml (treatment B) and 10^6 CFU / ml (treatment C) showed normal feeding response at day 6 observations until the end of observation (day 14). In contrary, fish infected with a density of 10^7 CFU / ml (treatment D) showed a positive response to feed normally from day 8 to the end of the

observation.

Based on the above description, it was shown that infected goldfish with lower bacterial density of 10^5 CFU / ml (treatment B) and 10^6 CFU / ml (treatment C) would gave normal feeding response faster than treatment D (10^7 CFU / ml) and E (10^8 CFU / ml) which returned to the normal condition and experienced high percentage of mortality (treatment E). This suggested that the higher density of bacteria which infected into the fish body, the longer feeding response of fish returned normally. Conversely, the lower the density of infected, the faster the fish would recovered to normal condition.

3.3 Shocks response

Based on the results of the observation of response to the shocks showed a different response on each treatment. The test fish in treatment A were not experienced a change in response to the shocks. All test fish infected with *A. hydrophila* on day 1 observation had decreased response to shock (Table 3).

Table 3: Shocks response after injection with *A. hydrophila*

Day-	Shocks response				
	A (Control)	B (10^5 cfu/ml)	C (10^6 cfu/ml)	D (10^7 cfu/ml)	E (10^8 cfu/ml)
1	++	-	-	-	-
2	++	-	-	-	-
3	++	+	+	-	-
4	++	++	++	+	dead
5	++	++	++	+	dead
6	++	++	++	+	dead
7	++	++	++	++	Dead
8	++	++	++	++	Dead
9	++	++	++	++	Dead
10	++	++	++	++	Dead
11	++	++	++	++	Dead
12	++	++	++	++	Dead
13	++	++	++	++	Dead
14	++	++	++	++	Dead

*Shock response observed during the experimental period indicated by No Response (-), Small Response (+), and Normal Shocks Response (++)

In Table 3, it showed fish in treatment B, C, D, and E on days 1 and 2 were not responded to the given shock (-). This happened because the fish were in a state of illness due to the attack of *A. hydrophila* bacteria. The absence of response to shock started with slow fish movement, some fish swam close to the surface or in the vicinity of aeration and swam with a sloping body position due to reduce the body equilibrium power. This results consistent with Affandi and Tang's (2002) statement that the characteristics of stressful fish are always on the surface of the water in a vertical position. According

Observations on day 3 in B and C treatments, fish started to respond to the given shock, but categorized as small responsive (+). Fish started to move slowly away from the source of shocks. The fish on treatment D and E were not responded to the shock until day 3 after injection. On day 4 after injection, the goldfish in treatment B and C showed a normal response (++), whereas in treatment D, small response occurred until day 6 and returned to normal at day 7 after injection. Control fish (treatment A) showed a normal response from the beginning of the observation to the end of the observation. Fish on treatment E (10^8 CFU /ml) from day 1 to day 3 had no response to feed, and all

fish died on day 4.

Infected test fish with low bacterial density (treated B and C), showed normal behavior returning faster, while test fish on D treatment (10^7 CFU / ml) recovery response to shock more slowly. Based on the above description, considering the higher the level of bacterial density that is infected into the fish body, the longer the recovery process of the test fish will return to normal. Novriadi *et al* (2014) [11] explains that behavioral changes and decreased reaction response to stimuli, weak fish responses, irregular swimming fish and skin discoloration which were clinical symptoms of fish infected by pathogenic bacteria.

The results showed that the goldfish infected by *A. hydrophila* bacteria had decreased feed response, shocks response, and severed inflammation, hyperemia, ulcers, ulcers, gill changes, and abscesses. This results in accordance with the results of research by Sarjito and Prayitno (2014) [15] that the clinical symptoms of carp infected by *A. hydrophila* were decreased feeding response, swimming around aeration, there were cuts and flesh of damaged fish in the form of ulcers and ulcers known as necrosis.

3.4 Mortality and survival of goldfish

Based on the mortality data of the goldfish for 14 days after being infected with *A. hydrophyla* showed the time and number of death varied depended on bacteria density (Table 4).

Table 4: Fish Mortality after Infected with *A. hydrophyla*

Day- (Hour-6)	Number of Mortality				
	A (Kontrol)	B (10 ⁵ cfu/ml)	C (10 ⁶ cfu/ml)	D (10 ⁷ cfu/ml)	E (10 ⁸ cfu/ml)
0	0	0	0	0	3
1	0	0	0	4	13
2	0	0	2	3	3
3	0	1	1	2	0
4	0	0	0	0	0
5	0	0	0	0	0
6	0	0	0	0	0
7	0	0	0	0	0
8	0	0	0	0	0
9	0	0	0	0	0
10	0	0	0	0	0
11	0	0	0	0	0
12	0	0	0	0	0
13	0	0	0	0	0
14	0	0	0	0	0
Total (fish)	0	1	4	9	19
SR (%)	100	95	80	55	5

Fish in treatment A or without infection *A. hydrophyla* showed no mortality recorded during the experimental period (SR 100%). Fish infected with *A. hydrophyla* bacteria with different density resulted in varying mortality and survival rate (Table 4 and Figure 6).

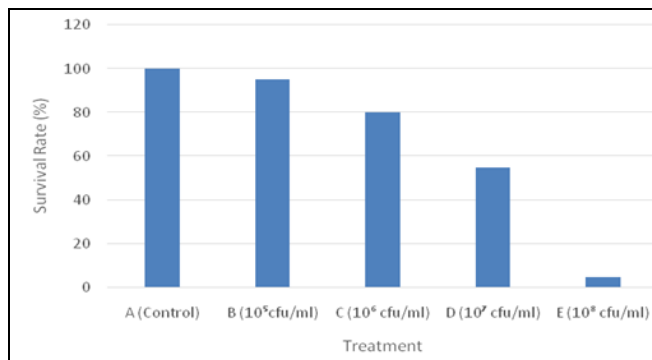


Fig 6: The survival rate of goldfish after infected by *A. hydrophyla* bacteria

Goldfish infected with *A. hydrophyla* bacteria showed varying survival rate, where the higher density resulted lower survival rate (SR), the lowest SR (5%) occurred in treatment with infected by of 10⁸ cfu / ml (treatment E) with the fastest starting time of death at 6 o'clock (Table 4) and the worst damage to the body (Table 1) after the infection. The low survival rate in treatment E was due to the infected fish decreasing the immune system and its metabolism has been disrupted. This showed the density of *A. hydrophyla* bacteria at that concentration is categorized as a virulent bacterial density. This results agree with Murwantoko *et al.* (2013) [10] that bacteria could categorized as a virulent if it could killed the fish more than 50% (LD 50> 50%).

The SR of goldfish on treatment B (10⁵ CFU / ml) and C (10⁶ CFU / ml) were relatively high, 95% and 80% with the time of early death were not so fast compared with treatment E, on day 3 for treatment B and day 2 on treatment C (Table 4). This showed that the concentration of *A. hydrophyla* at this concentration were not virulent (LD 50> 50%).

The goldfish on treatment D (10⁷ CFU / ml) resulted the survival rate of 55%, the value was close to LD50 and the time of early death was faster than treatment B and C, for example on day 1 (Table 4), so bacteria at this level was categorized as close to virulent categories.

The goldfish infected with *A. hydrophyla* bacteria which survived during the study were caused by the innate immunity of the fish itself, in accordance with Austin and Austin (1999) [2] stated that the surviving *A. hydrophyla*-infected fish had a high titer serum of antibodies such as IgM.

The mechanisms of fish mortality due to *A. hydrophyla* attack occurred because of the pathogenic *A. hydrophyla* bacteria producing endotoxins and exotoxins which include hemolysine and protease. Hemolysine is an enzyme capable of lysing red blood cells and liberating its hemoglobin, while protease is proteolytic enzymes that work against the host's body's defense for disease progression and take up host nutrient supplies for breeding (Figures 2001).

4. Conclusions

Based on the research results can be concluded that the higher density of *Aeromonas hydrophyla* bacteria infected in the goldfish resulted more severe surface damage and higher mortality. *Aeromonas hydrophyla* with the highest density of 10⁸ CFU / ml (treatment E) infected in goldfish categorized as virulent. Further research are needed to know the effect of *Aeromonas hydrophyla* bacterial density on histopathology condition of goldfish.

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