Assessing the susceptibility of the selected gourami (Osphronemus goramy) to Aeromonas hydrophila

RITA FEBRIANTI1,2,*, IKHSAN KHASANI3, KEKEU KANIAWATI ROSADA1,4,5
1Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran. Jl. Raya Bandung-Sumedang Km. 21, Jatinangor, Sumedang 45363, West Java, Indonesia. Tel./fax.: +62-22-7796412, *email: ritai19002@mail.unpad.ac.id, 5email: kekeu@unpad.ac.id
2Research Institute for Fish Breeding, Ministry of Marine and Fisheries. Jl. Raya 2 Sukamandi, Subang 41263, West Java, Indonesia

Abstract. Febrianti R, Khasani I, Rosada KK. 2021. Assessing the susceptibility of the selected gourami (Osphronemus goramy) to Aeromonas hydrophila. Nusantara Bioscience 13: 111-120. A breeding program to improve the growth performance of the gourami fish was carried out through selection methods that produced faster growth gourami (selected population). The purpose of this study was to determine the susceptibility of the selected gourami to Aeromonas hydrophila infection based on tolerance limits (LD50) and investigated clinical signs post-injection of the pathogenic bacteria. The challenge test by intramuscular injection of A. hydrophila was done to the fingerling fish (15.20 g) for 14 days post-infection. The population of the tested fish was obtained from six families, selected gourami (SP), and non-selected control (CP) groups. Phosphate buffered saline (PBS) as control and several doses of the pathogen, 105, 106, 107, and 108 CFU/mL of A. hydrophila, were injected into the fish. Fish mortality and clinical signs were observed daily. The fish mortality was confirmed by isolating bacteria in the fish which showed clinical signs, followed by biochemical characterization of the isolated bacteria using API 20E and PCR. The LD50 of A. hydrophila to the selected population (9.70 × 106 CFU/mL) was higher than that of the control (6.50 × 105 CFU/mL). On the final day of the test, the accumulation mortality of CP (63.33±5.77%) higher than that in SP (33.33±5.77%). Based on the output test statistics, it was known that there was a significant difference between the mortality and clinical signs of SP and CP. The data suggested that the selected gourami were more resistant to A. hydrophila infection than that of the control. The A. hydrophila infection caused most of the major clinical signs, including mass mortality of the fish. The biochemical and PCR test ensure that fish mortality was caused by A. hydrophila infection.

Keywords: Aeromonas hydrophila, challenge test, clinical signs, fish breeding, selected gourami

INTRODUCTION

Giant gourami (Osphronemus goramy Lacepede 1801) is an Indonesian native fish with high economic value. The national productivity of the gourami consistently increased, from 35.09% in 2015 to 198.97% in 2017, and 133.48% in 2018 (Ministry of Marine Affairs and Fisheries 2018). Therefore, the intensive farming of the fish has a high prospect to be developed. However, the slow growth character of the gourami is a serious problem to develop industrial farming of the fish. Disease attack is one of the obstacles in fish farming. Diseases can be caused by bacteria, viruses, parasites, and fungi. Bacterial disease in fish had been known since 1980. Aeromonas hydrophila bacteria causes Motile Aeromonad Septicemia (MAS) disease in gourami (Öztürk and Altinok 2014; Kusdarwati et al. 2018). Aeromonas hydrophila bacterial infection causes death and reduces the quality of the harvested fish (Purwaningsih et al. 2014). MAS disease is reported to cause mass mortality of cultured gourami in Indonesia. In 2016, the disease caused the loss of 47 tons of the gourami in West Sumatra (Department of Marine Affairs and Fisheries, Padang Pariaman Regency 2015), and mortality of 87-100% of the gourami population in the Banyumas area, the district of Central Java (Khumaidi and Hidayat 2018).

Intensive fish farming has a high prospect to be developed. Even though, the prevalence of disease outbreaks is relatively higher in this system. One of the major diseases in freshwater fish farming is Motile Aeromonad Septicemia (MAS) causing by A. hydrophila. The pathogenic bacteria cause tissue damage (invasiveness) and produce toxins (toxigenic) (Fernandez and Figueras 2020). The attack of A. hydrophila bacteria causes several clinical signs. Clinical signs are indications of the presence of a disease in the form of disease characteristics. According to Rozi et al (2018), the clinical signs of A. hydrophila bacteria attack is the presence of septicemic hemorrhage which is characterized by wounds on the body's surface, gills, ulcers, abscesses, exophthalmia, and flatulence. A. hydrophila bacteria cause disease outbreaks in catfish (Clarias sp.) (Kusdarwanti et al. 2017), tilapia (Oreochromis niloticus) (Hardi et al. 2018), gourami (Osphronemus goramy) (Rozi et al. 2018), and snakehead fish (Ophicephalus striatus) (Rao and Benarjee 2016). The determination of the observation period is based on the statement of Kusumawaty et al. (2016), that A. hydrophila bacterial infection in gourami is acute.

In the context of prevention and control of MAS disease, several strategies alternative has been done, including the management of integrated fish health, the use of MAS-free fish, and the vaccination (Ma et al. 2019). On the other hand, the specific pathogen resistance (SPR) of several cultured fishes has been developed in the world. Nevertheless, the SPR of MAS disease is not yet be
reported. Based on our observation in the gourami population, there were wide variations in the resistance level of the fish to bacterial disease. Refer to Hendry et al. (2011), the high variation of a specific character (disease resistance) in the population suggested that a selection method may be conducted to improve the character. In the natural disease outbreak, there were 10-20% of fish survive (survivor) (Subhan et al. 2020). Furthermore, the survivor can establish a based population to develop the SPR population (Moss et al. 2012). Based on the previous studies in the cultured species, selective breeding to produce MAS-resistant gourami may be conducted. Selective breeding for increased disease resistance is a promising strategy that has not been widely used in aquaculture. At the same time, improving growth performance is critical for efficient production (Hua et al. 2019). A study about the susceptibility of selected gourami, the specific strain of Indonesia, to A. hydrophila is not yet to be conducted.

The breeding program to improve the growth character of the gourami has been conducted in Indonesia for several years. The breeding program was done through selection, hybridization, and genetic engineering (Nugroho et al. 2012; Sularto et al. 2016; Arifin et al. 2017). The main purpose of selection is to change the quantitative phenotype of the population mean by exploiting genetic additives that have a beneficial trait from elders to tillers. For this purpose, the base population with high genetic variation is important to produce superior varieties. Several years of breeding programs to improve the growth character of gourami had been conducted and resulted in a selected population. Besides growth, resistance to disease and tolerance to pond conditions are important factors to aquaculture productivity (Reid et al. 2019). Therefore, a study to evaluate the susceptibility level to the disease on the selected gourami was conducted. L\text{D}_{50} is the amount of a toxic agent (such as a poison, virus, or radiation) that is sufficient to kill 50 percent of a population of animals. The purpose of the recent study was to evaluate the resistance level of the selected gourami, based on tolerance limits (L\text{D}_{50}) and investigated clinical signs post-injection of the pathogenic bacteria.

**MATERIALS AND METHODS**

**Experimental location**

This research was conducted in February 2020 at the research facilities of the Research Institute for Fish Breeding, West Java, Indonesia, including the gourami hatchery, The Microbiology Laboratory, The Genetics Laboratory, and The Water Quality Laboratory. The Research Institute is located at latitude 6°22’20.6”S 107°37’18.5”E, and an altitude of 25 meters above sea level (GPS Coordinates 2017).

**Procedures**

*Research design*

A total of 420 fingerlings were used for both the selected gourami (SP) and non-selected control (CP) groups. The SP and CP groups were chosen because the SP group was the result of the selection that had faster growth than the CP group. In addition, disease resistance is a promising strategy that has not been widely used in cultivation. The healthy gourami fingerlings weighed 16.2±2.12 g of body weight and measured standard length 9.80±0.48 cm. A completely randomized design was used in this study with seven treatments and three replicates. The treatments were infection of A. hydrophila to the gourami in several doses:

(i) **SPP:** Selected gourami infected with Phosphate Buffered Saline (PBS)
(ii) **SP-10²:** Selected gourami infected with 10² CFU/mL of A. hydrophila
(iii) **SP-10⁴:** Selected gourami infected with 10⁴ CFU/mL of A. hydrophila
(iv) **SP-10⁶:** Selected gourami infected with 10⁶ CFU/mL of A. hydrophila
(v) **SP-10⁸:** Selected gourami infected with 10⁸ CFU/mL of A. hydrophila
(vi) **SP-10¹⁰:** Selected gourami infected with 10¹⁰ CFU/mL of A. hydrophila
(vii) **SP-10¹²:** Selected gourami infected with 10¹² CFU/mL of A. hydrophila
(viii) **CPP:** Control gourami injected with Phosphate Buffered Saline (PBS)
(ix) **CP-10²:** Control gourami infected with 10² CFU/mL of A. hydrophila
(x) **CP-10⁴:** Control gourami infected with 10⁴ CFU/mL of A. hydrophila
(xi) **CP-10⁶:** Control gourami infected with 10⁶ CFU/mL of A. hydrophila
(xii) **CP-10⁸:** Control gourami infected with 10⁸ CFU/mL of A. hydrophila
(xiii) **CP-10¹⁰:** Control gourami infected with 10¹⁰ CFU/mL of A. hydrophila
(xiv) **CP-10¹²:** Control gourami infected with 10¹² CFU/mL of A. hydrophila

*Fish and acclimatization*

The population of the tested fish was obtained from six families, each for SP and CP. All the families resulted from one spawning batch in the controlled spawning pond of the gourami in the Research Institute for Fish Breeding. The fish were healthy and were free from A. hydrophila. Before the experimental tests, the fish were acclimatized for 14 d in fiber tanks (200 L capacity) under laboratory conditions. According to the research design, the normal fish, based on behavior, feeding response, and health status, were distributed in the 40 L of glass aquariums according to the research design.

Aeromonas hydrophila and preparation of the culture medium

*Aeromonas hydrophila* bacteria (American Type Culture Collection 35654 (ATCC 35654) was obtained from National Reference Laboratory the Biosafety in Microbiological and Biomedical Laboratories from the U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institute for Health, belonging to the Collection of Bacterial
Enteropathogens and maintained in cryopreservation. The liquid culture medium *Tripton Soya Broth* (TSB-Oxoid, England) was prepared according to the instructions of the manufacturer by dissolving 30 g in 1 L of distilled water. The sterilization was processed by autoclaving at 121°C for 15 min (Daikan Scientific Co., Ltd, Korea). The RS medium base (RS agar-Himedia, Mumbai) was prepared according to the manufacturer's instructions by dissolving 45.43 g in 990 mL of distilled water and it should not be sterilized using an autoclave. After removal and cooling between 45-50 °C, one vial of Novobiocin Supplement (FD096) was added followed by homogenization and distribution to the sterile Petri plate.

**Preparation of A. hydrophila suspensions**

Bacterial suspensions were prepared by transferring a heave containing three to five colonies of *A. hydrophila* isolated in Petri dishes containing the RS agar medium after 24 h of cultivation at 31°C to tubes containing 10 mL of BHI broth and reincubated for 18 h in a bacteriological incubator (Memmert, Germany). After incubation of the bacterial suspensions with the culture, the logarithmic growth phase was measured by turbidity caused by bacterial growth; Spectrophotometer (Hitachi U1500, Japan) was designed to measure the optical density of a suspension of microorganisms. A bacterial growth test was carried out by growing a bacterial isolate from an agar slant, then inoculating one loop of 30 mL TSB medium and incubating it for 24 h at room temperature. Inoculation of the results of liquid culture on TSB media into new TSB media in a test tube of 2 mL of isolate on 8 mL of liquid TSB. Incubation at room temperature and measuring OD (optical density) with a spectrophotometer at a wavelength of 620 nm every two hours for 30 h (Zubaidah et al. 2019). Before being used as a pathogen test, *A. hydrophila* bacteria was restored through the test of Postulate Koch on SPF gourami varieties. The test of Postulate Koch was carried out twice. Furthermore, this study was carried out by injecting 0.1 mL of inoculum into the test gourami intramuscularly (IM) (Taukhid et al. 2016).

**Susceptibility study of the gourami to A. hydrophila**

For the pathogenicity test, 420 gourami fishes were introduced into 42 research containers with 60 L water, and *A. hydrophila* was added intramuscularly injected of fish with PBS, 10^2, 10^4, 10^6, 10^7, and 10^8 CFU/mL. The mortality rate of the fish was recorded more than 24 h post-infection, and LD50 was determined. LD50 was estimated using the Dregsted Behrens method (Maryadi 2011). This study was carried out according to the principles adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Committee on the Ethics of Animal Use (CEUA/UFAC: 08/2014).

**Clinical signs of the gourami after A. hydrophila infection**

The symptoms of infection were observed, and after 24-96 h. The observations and measurements were carried out every 24 h. The mortality rate of the fish was determined at the end of the study. Water quality parameters including temperature, pH, dissolved oxygen, ammonia, nitrite, and nitrate were monitored at the beginning and end of the study. Observation of the clinical signs of gourami was carried out every 14 days (Taukhid et al. 2016).

**Confirmation test of the fish death**

The confirmation test aims to confirm the death of the gourami was caused by *A. hydrophila* bacteria. This test is performed by isolating bacteria from the liver, spleen, and kidneys, then purifying and characterizing it biochemically (API 20 E kit) and molecularly (PCR). The PCR method used is a method developed by (Alpha DNA Montreal, Quebec) using primary pairs of 16S F 5'GGG AGT GCC TTC GGG AAT CAG A 3' and 16S R: 5'TCA CCG CAA CAT TCT GAT TTG -3' with amplification of preheating temperature of 94°C for 2 min, denaturation temperature of 94°C for 1 min, the annealing temperature of 56°C for 30 sec, extension temperature of 72°C for 45 sec, final extension temperature of 72°C for 5 min with 30 cycles (Hussain et al. 2013). Furthermore, the PCR results were run by electrophoresis for 35 min with a voltage of 60 V and 400 A with a 1.5% agarose gel medium. The PCR product is seen in the High-Performance Ultraviolet Transilluminator with an ampiclon target of approximately 356 bp. The data was processed with three replications of the measured data and analyzed descriptively.

**Data analysis**

Water quality data were reported as mean ± SD. Observation of the clinical signs of the gourami was carried out every day by descriptive analysis to compare between groups. Water quality cultivation was used as a supporting parameter. Clinical signs were analyzed with a Mann-Whitney u test using SPSS. The data LD50 obtained from the research observation was analyzed using two-way ANOVA followed by Tukey’s post hoc test to analyze data sets. The confirmation test was done to confirm the death of gourami is caused by *A. hydrophila*.

**RESULTS AND DISCUSSION**

**Susceptibility study of the gourami to A. hydrophila**

In aquaculture, disease outbreaks in aquaculture are affected by three factors, such as host, pathogen, and environment. This study evaluated several doses of the pathogenic bacteria (*A. hydrophila*) and two populations of the gourami in the optimal condition.

The susceptibility of SP to *A. hydrophila* was indicated by 100 % mortality at a dose of 1 × 10^8 CFU/mL after 44 h. Different *A. hydrophila* susceptibility seen in CP was indicated by 100 % mortality at a dose of 1 × 10^6 CFU/mL and 1 × 10^7 CFU/mL after 24 h. The *A. hydrophila* bacterial at a dose of 1 × 10^2 CFU/mL did not cause the death of the SP and CP. The LD50 value of *A. hydrophila* to the CP, and SP were 6.50×10^5, and 9.70×10^4 CFU/mL respectively. The result of the analysis shows that F arithmetic is bigger than the F table (5 % and 1 %). Pairwise testing between SP and CP was performed by treatment when a significant interaction was found and multiple comparison adjustment was made using Tukey’s
method. There was a significant (p < 0.05), with a calculated F value of 96.100 > 4.35 F table, it can be concluded that there were differences in mortality results based on the type of population and the concentration of infected bacteria. There was a significant (p < 0.05), with an F count of 146.650 > 2.87 F table, it can be concluded that there were differences in mortality results based on the type of gourami population. There was a significant (p < 0.05), with an F value of 14.350 > 2.87 F table, it can be concluded that there was an interaction based on the type of gourami population and the concentration of bacteria injected in determining mortality in gourami. This indicates that the A. hydrophila batch-test conducted for 14 days in gourami had a significant effect on the susceptibility of gourami. The mortality of the SP and CP during the LD₅₀-96h of A. hydrophila is presented in Table 1.

Clinical signs of the gourami after A. hydrophila infection

In addition to causing death in the test fish, bacterial infection with lower doses can cause physiological disturbances which are indicated by several clinical symptoms, including loss of balance (LB), loss of appetite (LA), weakness (W), ulcers (U), necrosis (N), and hemorrhagic (H). Based on the output test statistics, it was known that the Asymp. Sig. (2-tailed) <0.05 for parameters LB, LA, W, U, N, and H. Thus there was a significant difference between the clinical signs of SP and CP. CP clinical symptoms were severe than SP. The clinical signs of the SP and CP post A. hydrophila infection presented in Table 2.

The early clinical signs that arise in this study were loss of balance or buoyancy control, floating upside down, or ‘sitting’ on the tank floor (most fish are normally only slightly negatively buoyant and take little effort to maintain the position the water column). As the disease progresses, the following may be observed: change in coloration, cloudy skin (indicates excess mucous production due to an irritant bacterial. Several hours after A. hydrophila injection, several clinical signs were observed in the infected gourami presented in Figure 1.

The bioassay pathogenic test must be conducted at the optimum level of water quality parameters to certain that the pathogen causes fish mortality. Refer to (Republic of Indonesia 2017), the main water parameters of the treatment tanks during this study were maintained at the optimum level for the giant gourami. They were 28.1-29.0°C of temperature, 8.00-8.42 of pH, 5.9-8.5 mg/L of DO, 0.0020-0.0163 mg/L of ammonia, 0.0097-0.0540 mg/L of nitrite, and 0.0430-0.9634 mg/L of nitrate. Based on the optimum level of water quality, the A. hydrophila infection causes the mortality of the fish during the recent study. The water quality parameters during this study were presented in Table 3.

Confirmation test of the fish death

Confirmation of fish mortality is carried out to ensure death due to the bacterial infection. All SP and CP fish were isolated from the dead fish from the liver, kidney, and spleen organs. As many as 100% of all SP and CP fish were isolated from the dead fish from the organs of the test results from the fermentative test, catalase test, gram stain test, PCR test, and API 20 E test showed that A. hydrophila caused the death of gourami. The fermentative test showed the color of the media turned yellow (Figure 2.A-B). The catalase test on bacteria that caused clinical signs showed positive results due to the formation of bubbles (Figure 2.C-D). Pure isolated bacteria were then stained and the result was that the bacteria were rod-shaped, short-chain, and red when tested with gram stain (Figure 2.E). The results of the PCR and API 20 E tests showed that fish mortality was caused by infection with A. hydrophila bacteria (Hussain et al. 2013). The results of confirmation bacterial isolation A. hydrophila of liver, kidney, and spleen organs of fish showed a band around 356 bp presented in Figure 3. Tests using API 20 E showed very good identification results with a percent ID value of 99% presented in Table 4.

Discussion

The LD₅₀ use of host death provides a nonequivocal endpoint and measurement has the advantage that it allows comparisons across microbes (Casadeval 2017). LD₅₀ figures are frequently used as a general indicator of a substance’s acute toxicity. A lower LD₅₀ is indicative of increased toxicity (Aisiah et al. 2020). Growth and survival are the major character in the aquaculture system and most selection programs focused on these traits (Krishna et al. 2011; Gjedrem et al. 2012). Based on these data, the LD₅₀ value of A. hydrophila to the control, and selected gourami were 6.50×10⁶ and 9.70×10⁶ CFU/mL, respectively. At this time, the average mortality in the control gourami had reached 63.33±5.77% and was significantly higher (p < 0.05) compared to that of selected gourami (33.33±5.77%). After 96 h post-infection, the gourami in both the control and selected groups showed normal behavior.

Table 1. Mortality of the selected gourami (SP) and control (CP) during the LD₅₀-96h of Aeromonas hydrophila

<table>
<thead>
<tr>
<th>Pathogen concentration (CFU/mL)</th>
<th>Number of fishes (0 h)</th>
<th>Number of dead fishes (96h)</th>
<th>Mortality (%)</th>
<th>LD₅₀ (CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPP</td>
<td>30</td>
<td>0</td>
<td>0.00±0.00</td>
<td>9.70×10⁶</td>
</tr>
<tr>
<td>SP-10²</td>
<td>30</td>
<td>0</td>
<td>0.00±0.00</td>
<td></td>
</tr>
<tr>
<td>SP-10⁶</td>
<td>30</td>
<td>25</td>
<td>16.67±0.58</td>
<td></td>
</tr>
<tr>
<td>SP-10⁵</td>
<td>30</td>
<td>23</td>
<td>23.33±5.77</td>
<td></td>
</tr>
<tr>
<td>SP-10⁶</td>
<td>30</td>
<td>20</td>
<td>33.33±5.77</td>
<td></td>
</tr>
<tr>
<td>SP-10⁷</td>
<td>30</td>
<td>14</td>
<td>53.33±5.77</td>
<td></td>
</tr>
<tr>
<td>SP-10⁸</td>
<td>30</td>
<td>30</td>
<td>100.00±0.00</td>
<td></td>
</tr>
<tr>
<td>CPP</td>
<td>30</td>
<td>30</td>
<td>0.00±0.00</td>
<td>6.50×10⁴</td>
</tr>
<tr>
<td>CP-10²</td>
<td>30</td>
<td>1</td>
<td>3.33±5.77</td>
<td></td>
</tr>
<tr>
<td>CP-10⁴</td>
<td>30</td>
<td>7</td>
<td>23.33±5.77</td>
<td></td>
</tr>
<tr>
<td>CP-10⁵</td>
<td>30</td>
<td>13</td>
<td>43.33±5.77</td>
<td></td>
</tr>
<tr>
<td>CP-10⁶</td>
<td>30</td>
<td>19</td>
<td>63.33±5.77</td>
<td></td>
</tr>
<tr>
<td>CP-10⁷</td>
<td>30</td>
<td>30</td>
<td>100.00±0.00</td>
<td></td>
</tr>
<tr>
<td>CP-10⁸</td>
<td>30</td>
<td>30</td>
<td>100.00±0.00</td>
<td></td>
</tr>
</tbody>
</table>
Pairwise testing between SP and CP was performed by treatment when a significant interaction was found and multiple comparison adjustment was made using Tukey’s method. Based on the output test statistics, it was known that the significant <0.05 for mortality. Thus there was a significant difference between the mortality of SP and CP. The susceptibility of CP was higher than SP. On the final day of the test, the accumulation mortality of CP had reached 63.33±5.77%, which was considerably higher than that in SP (33.33±5.77%) (Tabel 1). Fish susceptibility is manifested in fish mortality. Based on Table 1, the highest mortality occurred at a bacterial dose of 10^8 CFU/mL of 100%. Determining of clinical signs was done by observing the wounds on the outside of the body and the behavior of the gourami seeds infected with A. hydrophila bacteria.

![Figure 1](image1.png)

**Figure 1.** Clinical signs in the *O. goramy* were intramuscularly injected with *Aeromonas hydrophila*: A. Ulcers, B. Injury on caudal fin with hemorrhagic foci, C. Swimming at the bottom, D. Lesion at the site of *A hydrophila* inoculation, E. Weakness, and F. Integumentary injuries with depigmentation and hemorrhagic foci

![Figure 2](image2.png)

**Figure 2.** Confirmation of clinical signs and fish mortality by detection of bacteria: A. OF test, B. Test fermentative (TSIA), C. KOH test, D. Catalase test, and E. Gram stain test.
Table 2. The clinical signs of the selected gourami (SP) and control (CP) post *Aeromonas hydrophila* infection

<table>
<thead>
<tr>
<th>Observation time (hours)</th>
<th>Clinical signs</th>
<th>Footnote</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>LA-, W-, H-, N-, LB-, U-</td>
<td>LA: Loss appetite</td>
</tr>
<tr>
<td>24</td>
<td>LA+, W+, H-, N-, LB+, U+</td>
<td>H: hemorrhagic</td>
</tr>
<tr>
<td>48</td>
<td>LA+, W+, H+, N+, LB+, U+</td>
<td>NF: Necrosis</td>
</tr>
<tr>
<td>72</td>
<td>LA++, W+, H++, N++, LB+, U++</td>
<td>LB: Loss of balance</td>
</tr>
<tr>
<td>96</td>
<td>LA++, W++, H++, N++, LB+, U++</td>
<td>U: Ulcers</td>
</tr>
<tr>
<td>120</td>
<td>LA++, W++, H++, N++, LB+, U++</td>
<td>-: no symptoms</td>
</tr>
<tr>
<td>144</td>
<td>LA++, W++, H++, N++, LB+, U++</td>
<td>++: moderate</td>
</tr>
<tr>
<td>168</td>
<td>LA++, W++, H++, N++, LB+, U++</td>
<td>++++: severe</td>
</tr>
</tbody>
</table>

Table 3. Water quality conditions of the selected gourami (SP) and control (CP) during the LD<sub>50</sub>-96h of *Aeromonas hydrophila*

<table>
<thead>
<tr>
<th>Pathogen concentration (CFU/mL)</th>
<th>Temperature (°C)</th>
<th>DO (mg/L)</th>
<th>pH</th>
<th>Ammonia (mg/L)</th>
<th>Nitrate (mg/L)</th>
<th>Nitrate (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPP</td>
<td>28.7-29.0</td>
<td>8.2-8.5</td>
<td>8.02-8.20</td>
<td>0.0023-0.0132</td>
<td>0.0385-0.0540</td>
<td>0.0532-0.7850</td>
</tr>
<tr>
<td>SP-10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>28.4-28.9</td>
<td>7.4-7.7</td>
<td>8.05-8.10</td>
<td>0.0130-0.0123</td>
<td>0.0110-0.0234</td>
<td>0.0430-0.9634</td>
</tr>
<tr>
<td>SP-10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>28.5-28.7</td>
<td>6.9-7.0</td>
<td>8.40-8.42</td>
<td>0.0090-0.0130</td>
<td>0.0097-0.0172</td>
<td>0.0843-0.5870</td>
</tr>
<tr>
<td>SP-10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>28.2-28.6</td>
<td>6.0-7.1</td>
<td>8.02-8.30</td>
<td>0.0024-0.0130</td>
<td>0.0122-0.0242</td>
<td>0.0432-0.9630</td>
</tr>
<tr>
<td>SP-10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>28.1-28.8</td>
<td>6.1-6.4</td>
<td>8.22-8.37</td>
<td>0.0073-0.0090</td>
<td>0.0154-0.0340</td>
<td>0.3760-0.6260</td>
</tr>
<tr>
<td>SP-10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>28.2-28.6</td>
<td>6.0-6.3</td>
<td>8.10-8.21</td>
<td>0.0035-0.0086</td>
<td>0.0145-0.0321</td>
<td>0.1876-0.6589</td>
</tr>
<tr>
<td>SP-10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>28.2-29.0</td>
<td>5.9-6.3</td>
<td>8.00-8.10</td>
<td>0.0022-0.0163</td>
<td>0.0148-0.0367</td>
<td>0.1743-0.3398</td>
</tr>
<tr>
<td>CP</td>
<td>28.3-28.9</td>
<td>8.1-8.4</td>
<td>8.01-8.20</td>
<td>0.0026-0.0142</td>
<td>0.0388-0.0540</td>
<td>0.0532-0.7856</td>
</tr>
<tr>
<td>CP-10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>28.2-28.8</td>
<td>7.3-7.6</td>
<td>8.06-8.10</td>
<td>0.0128-0.0143</td>
<td>0.0114-0.0238</td>
<td>0.0432-0.9484</td>
</tr>
<tr>
<td>CP-10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>28.4-28.8</td>
<td>6.8-7.1</td>
<td>8.39-8.42</td>
<td>0.0092-0.0132</td>
<td>0.0104-0.0176</td>
<td>0.0840-0.5670</td>
</tr>
<tr>
<td>CP-10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>28.3-28.5</td>
<td>6.1-7.2</td>
<td>8.12-8.30</td>
<td>0.0028-0.0130</td>
<td>0.0126-0.0244</td>
<td>0.0532-0.9632</td>
</tr>
<tr>
<td>CP-10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>28.2-28.9</td>
<td>6.2-6.4</td>
<td>8.22-8.37</td>
<td>0.0073-0.0090</td>
<td>0.0154-0.0348</td>
<td>0.3840-0.6266</td>
</tr>
<tr>
<td>CP-10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>28.3-28.7</td>
<td>6.1-6.3</td>
<td>8.14-8.21</td>
<td>0.0038-0.0088</td>
<td>0.0148-0.0324</td>
<td>0.1882-0.7658</td>
</tr>
<tr>
<td>CP-10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>28.2-28.9</td>
<td>5.9-6.2</td>
<td>8.00-8.20</td>
<td>0.0020-0.0163</td>
<td>0.0152-0.0382</td>
<td>0.1842-0.3698</td>
</tr>
</tbody>
</table>

Threshold value

(Republic of Indonesia 2017)

25.0-30.0 > 3.0 6.50-8.50 ≤ 0.02 ≤ 0.06 ≤ 20

Table 4. Identification of bacteria using the API 20 E kit

<table>
<thead>
<tr>
<th>Reference: Very good identification</th>
<th>Aeromonas hydrophila/caviae/sobria 2</th>
<th>% ID</th>
<th>T</th>
<th>Test against</th>
</tr>
</thead>
<tbody>
<tr>
<td>Significant Taxa</td>
<td>99.0/1.0</td>
<td>0.69</td>
<td>0.129 R</td>
<td>Methyl Red</td>
</tr>
<tr>
<td>Next taxon</td>
<td>% ID T</td>
<td>0.69</td>
<td>0.129 R</td>
<td>Methyl Red</td>
</tr>
<tr>
<td>Complementary test(s)</td>
<td>Glucose g, ESC (HYD)</td>
<td>0.38</td>
<td>0.129 R</td>
<td>Methyl Red</td>
</tr>
<tr>
<td>Aeromonas caviae</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>86 %</td>
</tr>
<tr>
<td>Aeromonas hydrophila</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>86 %</td>
</tr>
<tr>
<td>Vibrio fluvialis</td>
<td>% NT</td>
<td>-</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>Aeromonas sobria</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>-</td>
</tr>
</tbody>
</table>
This study showed that the selected gourami was higher resistant than the control. Because, selected gourami had high genetic variation, it will provide a great opportunity to get the right combination of crosses with good disease resistance (Suprapto and Kairudin 2017). Selected gourami can be used as a candidate for superior gourami. According to Sularto et al. (2020), the growth of selected gourami in nursery and resistance phases was higher than that of the control. This corresponds to the results of the study of the LD50 value of selection gourami were higher than pure gourami (Sularto et al. 2020). High genetic variation will provide a great opportunity to get the right cross combination with a good combination of traits (Suprapto and Kairudin 2017). Based on the result, the selection program to increase resistance to gourami. On the other hand, Sularto et al. (2020) reported the SP groups had better growth than CP. Based on the result, the selection program to increase resistance to gourami did not negatively affect the growth of gourami. Therefore, a breeding program to improve the resistance level of the gourami to specific pathogen could be applied in the giant gourami. Besides that, other selection lines based on the growth character of the gourami have improved 11.18% of their productivity (Sularto et al. 2020).

The correlation pattern between disease resistance and growth is host-specific and pathogen-specific (Gjedrem 2015). On the other hand, Suebsong et al. (2019) reported the same thing selective breeding programs have significant potential to make tilapia more resistant to Streptococcus agalactiae. Heritability estimates for G0 were 0.22 using the Cox model. At the same time, the researchers noted that selection response indicated that the risk of death decreased to 54 percent, survival time increased to 3.4 days. Survival rate increased to 21%, suggesting that breeding tilapia that are more resistant to S. agalactiae is possible. On the other hand, faster growth also occurring in disease-resistant populations has been reported in several aquaculture species. Parker et al. (2011) reported that Line 1 grows at twice the rate of non-selected oysters, has a higher standard metabolic rate, and has a significantly higher survival when exposed to elevated levels of PCO2 and temperature predicted to occur as a result of climate change. Differences in reproductive status between fifth-generation fast growth oysters and non-selected oysters were found by Dove and O’Connor (2012). The authors are monitoring changes in reproductive condition in Lines 1-3 to determine if this also occurs in SROs bred for disease resistance. Huang et al. (2012) reported that that Pacific whiteleg shrimp having 21% higher resistance to WSSV grew 34.51% faster than that of the control. Another study showed that the growth of oyster resistant (Crassostrea gigas) to herpesvirus 1 (OsHV-1) was 15% faster than that of the control (Lionel et al. 2015). Furthermore, Khasani et al. (2017) reported that the growth of giant freshwater prawns (Macrobrachium rosenbergii) resistant to vibriosis was 46% higher than that of the control. The neutral correlation between growth and resistance to bacterial cold-water disease was also observed in rainbow trout. Besides that, the growth of Atlantic salmon (Salmo salar) resistance to Piscirickettsia salmonis and Caligus rogercresseyi was similar to that of the normal fish (Yanez et al. 2014). Another case reported that genetic correlations between body weights and WSSV resistance in Pacific Whiteleg Shrimp and Liptopeneaeus vannamei were negative (Trang et al. 2019).

The LD50 of A. hydrophila in this study is lower than in several previous studies. The LD50 of Bogor gourami was 105 CFU/mL (Taufkid et al. 2016), tilapia was 4.9×10⁶ CFU/mL (Mangunwardoyo et al. 2016), Arapaima gigas was 1.8×10⁶ CFU/mL (Dias et al. 2016), strain K14 in African catfish was 4.977×10⁵ CFU/mL (Wulandari et al. 2014), and strain ASB01 in snakehead was 2.69×10⁵ CFU/mL (Olga 2014). Citterio and Biavasco (2015) classify A. hydrophila into virulent and non-virulent bacteria. A. hydrophila isolates with an LD50 of 10⁴, 10⁵ CFU/mL were declared virulent, while A. hydrophila isolates with an LD50 of 10⁶ CFU/mL or more were declared non-virulent. The mortality in gourami infected with A. hydrophila proves that the bacteria are pathogenic and very virulent in fish. Characteristics of bacteria that are pathogens include transmissibility, adherence to host cells, persistence, invasion of host cells and tissues, toxigenicity, and the ability to evade or survive the host’s immune system (Jawetz et al. 2014). This difference is probably due to differences in serotype and biotype of bacteria, fish species, and temperature (Olga 2014). Besides that, the difference in test results and LD50 calculations is thought to be due to the very different sources of origin of bacteria and host fish used (Makrinos and Bowden 2016; Li et al 2017; Yengkhom et al. 2019). Changes in the physical and chemical characteristics of the environment can increase the abundance and virulence of pathogenic organisms as can genetic mutation, factors which must have an important influence on the outcome of a situation in which pathogens challenge fish in the water (Tipathi et al. 2018). However, another influence, namely the degree of susceptibility of the host, may also be instrumental in determining whether or not pathogenic challenge results in disease (Tipathi et al.

Figure 3. Gel electrophoresis of product using 16S F & 16S R primers, M denotes 100 bp DNA ladder (Marker), H1-H4: liver, G1-G3: kidney, L1-L3: spleen, (+): positive control, and (-): negative control

Olga 2014).
Therefore, disease predisposition in fish can be said to be the end result of an interaction between host susceptibility, pathogen virulence, and environmental factors (stressors) (Gjedrem 2015). Stress weakens fish immune systems, and increases susceptibility to disease (Abram et al. 2017).

Further clinical signs are the appearance of white spots on the body of the fish, mucus accumulation on the body, skin lesions, white pectoral fins, and red spots. Other clinical signs include a dorsal fin, injury to the injection site, and ulcers (Figure 1.A). Internal symptoms that arise due to A. hydrophila infection include the presence of yellow fluid in the abdominal cavity, pale red and tender kidneys, brownish-red liver, heart, gills, pale intestines, swollen stomach filled with water or necrosis of the fins and tail, ulcer (Figure 1.B). Changes in swimming patterns that occur after the fish are infected with A. hydrophila in the form of fish tend to be aggressive with a dorsal fin that is the weakness in the bottom of the aquarium (Figure 1.C). Cloudy skin (indicates excess mucous production due to an irritant bacterial (Figure 1.D) due to bacterial infection of the kidneys. Weakness on the surface of the aquarium (Figure 1.E), as well as a reddish color all over the fish's body (Figure 1.F). Aeromonas bacteria can attack fish fins, tegument, and intestines. According to Rozi et al. (2018), these bacteria are capable of rupturing little blood vessels, resulting in ulcerative lesions in the tegument with a hemorrhagic aspect, causing a reddish color on the body. In this study, besides the ulcerative lesions observed on the body, exophthalmia, and mucus excess were also observed. Rozi et al. (2018) have described these manifestations as clinical signs of A. hydrophila infection and, according to Oliveira et al. (2011), the high proliferation of these bacteria on fish intestine can cause excessive mucus liberation. Based on the output test statistics, it was known that the Asymp. Sig. (2-tailed) <0.05 for parameters LB, LA, W, U, N, and H. Thus there was a significant difference between the clinical signs of SP and CP. CP clinical symptoms were severe than SP.

Aeromonas hydrophila belongs to the group of pathogenic bacteria with high virulence. The virulence level of the bacteria is determined by the ability of bacteria to produce enzymes and certain toxins it plays a role in the invasion and infection process (Leitão 2020). In the first step, the pathogen attaches to the fish scales and produces the chitinase enzyme. The enzyme destroys the chitin of the scales layer. A further step, the bacteria enter the fish's body through the bloodstream. This process is assisted by the lecithinase enzyme which has a specific function to penetrate the bloodstream. Furthermore, the toxin will spread through the bloodstream (Andriani et al. 2020). The disease caused by A. hydrophila bacteria is called Motile Aeromonad septicaemia (MAS) because the infection spreads throughout the body through the bloodstream, infects fish through the surface of the body or wounded gills, and then enters the blood vessels and other internal organs (Ulfiana et al. 2012). According to Yengkhom et al. (2019), disease malignancy is influenced by interrelated factors, such as bacterial virulence, types of bacteria, and the degree of stress affected by fish populations, physiological conditions of the host, and the degree of genetic resistance that cannot be separated in specific populations of fish.

Haryani et al. (2012) also mentioned that the first reaction of animals in cellular and vascular to bacteria that enter the body that causes damage to tissue is inflammation (Table 2). This tissue damage is thought to occur due to toxins released by these bacteria and carried throughout the body through the bloodstream. This was also stated by Andriani et al. (2020), that the toxin was spread throughout the body through the bloodstream causes hemolysis and rupture of blood vessels resulting in redness or red spots on the body of the fish. The appearance of clinical signs can affect the appetite of fish. Fish experience changes in appetite after injection. According to Hardi et al. (2017), fish infected with bacteria take a longer time digesting food than uninfected fish. In infected fish, the feed condition is still intact at 5 min after giving. This is due to disruption of the digestive enzymes of the fish due to a bacterial infection of the brain that regulates intestinal peristalsis. Bacterial infection of the brain can inhibit the work of the lateral hypothalamus which regulates diet. If the hypothalamus which is in the telencephalon (forebrain) is infected, the fish appetite will decrease (Hardi et al. 2018). Based on Figure 3, gram staining results indicate that A. hydrophila bacteria were gram-negative, short rod-shaped, non-spore, motile, and have one flagellum, living in the temperature range 25-30°C (Yamazaki et al. 2021). A. hydrophila bacteria are aerobic and facultatively anaerobic. The 16S rRNA primer was used to amplify 356 bp. Fragment of the 16S rRNA gene is conserved for the genus Aeromonas to confirm the presence of Aeromonas spp. (Hussain et al. 2013).

Selected gourami that is resistant to disease and has a higher tolerance to environmental disturbances is a key factor to increase the survival in gourami farming. The average mortality in the non-selected control had reached (63.33±5.77%) and was significantly higher (p < 0.05) compared to that of selected gourami (33.33±5.77%). In conclusion, the result of this study demonstrated that non selected group was more susceptible than the selected groups.

ACKNOWLEDGEMENTS

The DIPA budget funded this study with the Number: SP DIPA-032.12.2.403882 / 2020. We thank Sularto, and laboratory technicians and the gourami team have helped a lot in completing data.

REFERENCES

Ophicephalus striatus

- SV).

ata

. Acta Amazon 41

Carassius auratus

, Osphronemus gourami

) for treatment of

eeding and biosecurity in the prevention of Q, Yan X, Li J, Li X

resistance to

Huang YC, Yin Z, Weng S, He J, Li S. 2012. Selective breeding and

Hary

Hardi EH, Nugroho E, Nafiqoh N, Gustiano R. 2012. Productivity of several


