Study on characterization, pathogenicity and histopathology of disease caused by *Aeromonas hydrophila* in gourami (*Osphronemus gouramy*)

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Abstract. This study aims to determine the bacterial pathogens that cause disease of the gourami in Blitar (East Java) and Yogyakarta (Central Java), Indonesia. A total of 50 fish samples taken randomly gourami in pond farmers in seventh different locations. There were 18 isolates were isolated and then test Koch's postulates were injected 0.1 ml/fish intraperitoneally to gourami. Characterization is done by using the biochemical tests. Pathogenicity test carried out on 3 isolates of *Aeromonas* spp. with intraperitoneal injection at a dose of $10^4-10^8$ CFU/ fish, the value of *Lethal Dosage 50* (LD50) using the method Dragstedt Behrens. After the treatment, spleen and kidney samples were processed for histopathological analysis. The all of identified bacteria were 5 isolates *Aeromonas hydrophila*. Isolates of *A. hydrophila* in a row AH3 was virulen to gourami with LD50 (4.53 x $10^6$ CFU/fish), while isolate AH4 and AH5 (2.903 x $10^8$, 1.319 x $10^9$ CFU/fish) not be avirulen. Koch's postulates; 3 isolates are pathogenic with mortality of 40-100 % and 2 are non-pathogenic isolates with a mortality of 0 %. Clinically; ulcers, haemorrhagic at the base of the fins, body, mouth and exophthalmia. Histopathologically indicated spleen necrosis, piknosis, necrosis and inflammatory cells in kidney.

1. Introduction

The giant gourami, *Osphronemus gouramy*, is an Indonesian native fish that has a widespread distribution in Southeast Asia [1,2,3]. It is a commercially important freshwater herbivorous species with a relatively high price [4,5]. Therefore *O. gouramy* is a commodity that is seeded in aquaculture and is an important species in the ornamental as well as edible aquaculture industries [6]. However, production has not been able to meet the market demand.

There are various ways to increase gourami production such as through intensive or extensive cultivation technology, but fish cultivation is prone to the presence of disease as a result of interactions between the host, the pathogen, and the environment [7]. Kamiso [8] stated that the three main barriers
in fish farming in Indonesia are inferior seed supplies, expensive feed supplies, and disease. Most of the cause of disease in fish is pathogenic bacteria which is more commonly known as bacterial disease, especially gram-negative bacteria such as Aeromonas sp., Vibrio sp., Pseudomonas sp., Edwarsiella sp., and Flexibacter sp. [9]. Outbreaks caused by Aeromonas hydrophila have resulted in high economical losses and the death of nearly 80-100 % of fish cultivated within 1-2 weeks [10]. Infection from the bacteria can show clinical signs of skin infection in wounds such as ulcers that extend to muscle tissue, fins, rotting gills, protruding eyes (exophthalmia), and swelling of the spleen and the kidneys [11].

Research on disease-causing bacteria found in gourami has been carried out in Bantul, Yogyakarta and Blitar, East Java and very important work has been done by isolating, identifying and inventorying the bacteria. It is hoped that through this research, we can obtain pure isolates and know the symptoms and its pathogenicity so that it can be used in the initial steps of the monitoring, prevention and handling of the onset of the bacterial plague which has widely caused disease in large freshwater fish/gourami.

2. Methodology

2.1. Sample fish
About 30 diseased gourami (O. goramy) were collected from five farm ponds in Bantul, Yogyakarta, Central Java (7°52’29.34”S, 110°19’31.93”E) and Blitar, East Java (8° 5’ 60.0000” S and 112° 9’ 0.0072” E.). The fish weighed from 350 to 800 g with the size of 90-10 cm. The fish were anaesthetised with clove oil and then dissected according to Wilson [12] and performed by standard methods [13].

2.2. Bacterial isolation
Lesions from the skin, fins and gills were inoculated onto Trypticase Soy Agar (TSA) and incubated aerobically at 28°C for 24 h. Then internal samples were acquired aseptically from the kidney, liver, spleen and infected muscles. The plates were examined for bacterial growth. Dominant colonies were selected and re-streaked on Trypticase Soy Agar (TSA). Cultures were placed in 20 % glycerol and supplemented in Trypticase Soy Broth (TSB) for storage at -80°C. Dominant colonies were selected for bacterial isolation to establish the optimal number of bacterial cells, and went through a purification procedure until pure colonies were established to be sure that the dominant colonies were not contaminated.

2.3. Identification of the isolates
Bacterial identification was done on the basis of Bergey’s Manual of Determinative Bacteriology [14], Biochemical tests for Identification of Medical Bacteria [15], then completed with Bacterial Fish Pathogens: Disease in Farmed and Wild Fish [9].

2.4. Pathogenicity test
Pathogenicity is determined based on value of LD50 using method Dragstedt Behrens [16]. The calculation of the density of the pathogenicity test is based on the method of counting the bacteria indirectly by the number of colonies that is total plate count (TPC) according to Junoto et al. [17].

2.5. Histopathological examination
For histopathological studies, tissue specimens were obtained from the liver and kidney. The tissue specimens were fixed in 10 % neutral buffered formalin. Dehydration and infiltration of tissue were carried out using an automatic tissue processor (Sakura, Japan). Samples were embedded in paraffin, and were sectioned using a rotary microtome (4 to 5 μm) (Sakura, Japan) and stained with Hematoxylin and Eosin (H&E) according to the method described by Carleton et al. [18].
3. Results and Discussion

3.1 Clinical signs

Gouramis weighing 50-135 grams or (13-20 cm) were obtained from five locations each both in Bantul, Yogyakarta, and Blitar, East Java, Indonesia. Almost the same symptoms were found on the gourami samples taken from each location (table 1).

Table 1. The origin of sampling, gross clinical and pathological symptoms.

| Origin of samples | Fish | Species weighed (gram) | Length (cm) | Symptoms |
|-------------------|------|------------------------|-------------|----------|
| Bantul, Yogyakarta, Center Java, Indonesia | 30 Goramy | 50-60 | 13-20 | Releasing air bubbles from the operculum, geripis fins, Often appeared on water |
| Blitar, East Java, Indonesia | 20 Goramy | 80-100 | 16-18 | Eyes were exophthalmia and reddish, torn fins hemorrhagic, Releasing air |

The symptoms that were generally seen include: the indication of the presence of air bubbles expelled through the operculum, huddled above the water surface, hemorrhagic in some parts of the body especially the base of fin. Some samples of the haemorrhaging were still visibly light, i.e. there were red smudges that were still separate, but other samples like the dorsal, pectoral and tails fins were torn and broken part. The oral cavity was reddish, one or both eyes exhibited exophthalmia and scales were loss in some of the samples; while internal symptoms displayed a change in the color of the kidney and had a tendency to swell. In general, it shows that the more severe the physical (external) symptoms, the more severe the organ (internal) symptoms were. Gourami samples can be seen in the sample (figure 1).

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Figure 1. A. Hemorrhage of around the abdomen. B. Typical morphology of A. hydrophila.

3.2. Bacterial isolation and identification

Out of the 50 samples of gourami from the five of ponds of different locations in Bantul and Blitar that were allegedly infected by bacteria, results of the biochemical test showed that 5 out of 18 A. hydrophila
isolates were successfully obtained. The bacteria that were pathogenic were found in three isolates (40-100 % mortality) and 2 isolates were non-pathogenic (0 % mortality). Biochemical tests were conducted on the identification of 18 isolates of A. hydrophila. About five isolates were identified as Gram negative (figure 1B), motile, oxidase-positive, catalase-positive, etc (table 2).

3.3. Virulence degree

The pathogenicity test of A. hydrophila in this study can be identified through the LD50 test devoted to the death of 50 % of the population due to infection of A. hydrophila. After that, results of the observations of LD50 of the bacteria dose resulting in death of 50 % of gourami was in the range of 10^5-10^7 CFU/ml. Through the calculation with the method of Bargstedt-Behrens, the LD50 value obtained from the AH3 isolates was measured at 4.53 x 10^6 and was categorized as virulent; while according to Austin & Austin [9] (table 3), the A. hydrophila non-virulent isolates were AH4 and AH5 with the LD50 values of 2.90 x 10^8, 1.31 x 10^9 respectively.

Table 2. LD50 values of the three isolates.

| Isolates | Bacteria     | LD50  (CFU/fish) | The degree of Virulence | Reference |
|----------|--------------|-----------------|------------------------|-----------|
| AH3      | A. hydrophila| 4.53 x 10^6     | Virulent*              | 9         |
| AH4      | A. hydrophila| 2.903 x 10^8    | Non Virulent*          | 9         |
| AH5      | A. hydrophila| 1.319 x 10^9    | Non Virulent**         | 9         |

3.4. Clinical signs and gross changes

Clinical findings observed from the 90 diseased gourami samples for the patogenicity test showed differences among the fish. Some diseased gourami showed clinical symptoms while others showed more than two clinical symptoms. These clinical symptoms include: skin lesions with hemorrhaging, ulcers, inflammation and hyperemia in the fins. There is a "washboard" on the skin, exophthalmia in one or both eyes, a histopathological section observed of liver and kidney; piknosis, necrosis liver and piknosis, necrosis, inflammatory cells kidney (figure 2).

Figure 2. Histopathological section of A: liver; (yellow row) piknosis, (blue row) necrosis cells (H&E stain 100X). B: Kidney; (blue row) piknosis, necrosis (yellow row) inflammatory cells (H&E stain 100X).
Table 3. Characters of bacterial strain AH1.

| Parameter Characters                        | Characters          |
|--------------------------------------------|---------------------|
| Colony Morphology                          |                     |
| Colony shape                               | Circulair           |
| Elevasy shape                              | Convex              |
| Edge shape                                 | Entire              |
| Oblique agar                               | Echinulate          |
| Colour                                     | cream               |
| Size                                       | 2 mm                |
| GSP/colour                                 | + (cream))          |
| Cell Morphology                            |                     |
| Gram                                       | -                   |
| Shape                                      | rod                 |
| Phenotype of biochemical                   |                     |
| O/F                                        | F                   |
| Motility                                   | +                   |
| Produksi                                   |                     |
| Katalase                                   | +                   |
| Oksidase                                   | +                   |
| H2S                                        | +                   |
| Lisin dekarboksilase                       | +                   |
| Arginine dehydrrolase                      | ND                  |
| Onitin dekarboksilase                      | -                   |
| TSIA                                       | K/A, G              |
| LIA                                         |                     |
| Growth in 30°C                             | +                   |
| Growth in NaCl 6.5 % Methyl red            | +                   |
| Voges-Proskauer                            | +                   |
| Sensitivity Novobiocin                     | +                   |
| Sensitivity 129.10                         | -                   |
| Simmon citrate                             | +                   |
| Gelatin broke                              | +                   |
| Acid production                            |                     |
| D - Glucose, acid, D - Galaktose, acid, Maltose | +           |
| D - Mannitol, D - Mannose, Sucrose, acid Inosito | -                |
| Dulcitol, Raffinose, D - Sorbitol, D – Xylose, Inulin | -             |
| Gas production - D - Glucose, gas          | +                   |
The samples of the 50 gourami fish allegedly infected by bacteria had the size of 13–20 cm. 18 isolates were successfully isolated from the samples from five different locations of both Bantul, Yogyakarta and Blitar, East Java. The samples in the field show symptoms of septicemia. The symptoms generally seen were in the form of hemorrhage, wounds such as sores, reddish wounds around the mouth, and exophthalmia eyes, but overall the samples showed such symptoms such as removed oxygen from the operculum, dark and pale samples, and mass death such as what occurred at the Bantul location where the mortality rate reached 100 % of 20,000 stocking densities cultured on the size of consumption. This is consistent with the literature stating that an *A. hydrophila* infection is acute when marked by the very quick death of the fish with the appearance of signs of clinical infection like exophthalmia, red patches on the skin, and accumulation of fluid in the abdominal pouch, flatulence, bleeding gill and injury to the dermis, and separated scales [20]. According to Kamiso, [21] *A. hydrophila* can cause the death of 90-100 % in a short time. Research by Daskalov [22] also described that *A. hydrophila* is widely distributed through food, drinking water and the environment, and is a pathogen that causes hemorrhagic diseases, zoonotic diseases, and food-borne infections in freshwater fish. Several factors are involved in the *A. hydrophila* virulence process. *A. hydrophila* is naturally found in the fish intestinal tract, and is not caused by factors such as: stress, changes in environmental conditions, population density, handling, transport, poor water quality, temperature changes, low dissolved oxygen levels, high CO₂ levels, high nitrite levels, and high ammonia levels. This is the most common predisposing factor associated with *A. hydrophila* disease. In addition, the pathogenicity of *A. hydrophila* appears to be related to how stressful the hosts are infected. *A. hydrophila* with high virulence can infect healthy fish; however, stress derived from intensive fish cultivation also contributes to it and can trigger an outbreak [23].

Overall 5 isolates were *A. hydrophila*. The results of biochemical tests are capable of utilizing gelatine as a substrate protein. Bacteria that can take advantage are albumin, casein, fibrinogen and gelatine as a substrate protein, so it can be inferred that strains of this bacteria are proteolytic [24]. According to Neito and Ellies [25], there are at least four or perhaps five kinds of protease extracellular of *A. hydrophila*. Based on data from the study of this biochemical test, *A. hydrophila* overall or 100 % produces proteolytic enzymes / enzyme gelatinase. When seen from the relationship symptoms, *A. hydrophila* allegedly caused the death of 40-100 % at 1-2 weeks [21] by utilizing proteolytic enzymes such as protease that functions as the body's defense against the host in the progression of the disease [26]. The internal organs of fish infected by *A. hydrophila* experience hemorrhaging indicated by the occurrence of hemolysis in vitro, so the hemolysin was also considered as a major virulence factor of *A. hydrophila* [27]. Judging from the distribution of the spreading of the bacteria, the one that is most commonly found by percentage (71.42 %) was *A. hydrophila* which was found in almost all sampling sites. One of the factors that cause the extent of the spread of the bacteria is that it is opportunistic in nature and capable of living outside the host in the long term.

The phatogenicity test showed that about 3 out of 5 *A. hydrophila* were virulent to gourami with LD50 (4.53 x 10⁶ CFU/fish), and Koch's postulates; 3 isolates were pathogenic with mortality of 40100 %. There are several factors involved in the *A. hydrophila* virulence process. In this study, the overall virulence factor of *A. hydrophila* isolates includes hemolysin and aerolysin. Aerolysin is the most common virulence factor found in the isolates analyzed [28]. The difference of the level of virulence in each isolate of *A. hydrophila* and *V. cholera* depends on the strain used, the age of the individual, the individual test conditions, and water quality. The onset of the disease is also very important with the factors of bacterial pathogenicity, the propagation speed of pathogens, as well as factors of the host defense against pathogens. Activities or leukosidin and haemolysin produced by the Extracellular products (ECPs) of bacteria become a bacterial defense factor against the defence of the host’s blood because it is able to lysis blood cells. The bacteria are able to survive and will follow the blood flow to spread
throughout the body of the host cells as well as towards the target organs. Bacteria also have pathogenicity factors in the form of the enzymes found in ECPs, including caseinase, gelatinase, amylase, lipase, phospholipase, chitinase, kolagenase, elastase, hyaluronidase, and proteinase. These enzymes are able to decipher the complex compounds into simpler compounds, so the bacteria can easily break through the host cell [29].

The kidneys and liver of fish are internal organs that are sensitive to aeromonas disease attacks. Results of histologic analysis of the kidneys and liver showed a quite significant difference between healthy gourami fish and those exposed to *A. hydrophila* (figure 2). In healthy fish, the slices of liver are brightly colored as well as the hepatocyte cells containing the nucleus and heterochromatin. The condition of liver cells in fish affected by an *A. hydrophila* attack appears damaged because it has an infection, but does not release pus (nonvirulent multifocal hepatitis). Picnosis, necrosis cells and cells blood due to internal bleeding (internal haemoragy) were also found. Death of liver cells (focal necrosis) is a common manifestation of fish affected by *A. hydrophila* [30]. Fish kidneys exposed to *A. hydrophila* show picnosis, inflammatory cells cell death (necrosis) caused by the enzymatic degradation produced by *A. hydrophila*, as stated by Burr et al. [31]. All of the gourami fish strains were stricken under the same pathogenic conditions based on histopathologic analysis of the renal organs and heart. This study showed relatively similar results with Afifi [32], which described that the toxins produced by *A. hydrophila* and extracellular products such as hemolysin, protease, elastase can cause severe necrosis in the liver. The study also observed that the diseased kidneys of the fish were severely damaged and showed degenerative changes in the glomerular epithelium with cytoplasmic vacuol formation, as well as focal lymphocyte infiltration. This is similar to Suprapto's report [33] which states that the kidney is attacked by bacterial toxins that cause kidney cells to lose its structural integrity.

During the past decades, the development of suitable vaccine(s) and vaccination strategies have contributed to controlling infectious diseases in aquaculture in respect to the type of pathogen and cultured species [34]. However, till date only a few vaccines, i.e., against enteric red mouth, furunculosis, vibriosis, pasteurellosis, yersiniosis, etc., are available [34]. These vaccines are mostly polyvalent, containing the whole cell antigen or components of a more pathogen. The major problem associated in immunizing individual fish with antigen is that it lives in an environment where a wide range of other pathogens and secondary invaders are present, and a specific immunization strategy may only be effective in protecting against a specific pathogen or disease.

4. Conclusion

These results indicate that the strains in the sample showed symptoms of a disease-causing bacteria which are generally manifested in the form of ulcers, exophthalmia, hemorrhaging at the base of the fin, body and mouth, and air bubbles from the operculum. The isolate of bacterial pathogens that cause disease in gourami have been identified as *A. hydrophila* and five isolates were obtained. The AH3 isolate of *A. hydrophila* is pathogenic to gourami with LD$_{50}$ (4.53 x 10$^6$ CFU/fish), while the isolates of AH4 and AH5 (2.903 x 10$^8$, 1.319 x 10$^9$ CFU/fish) is avirulent.

5. References

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