

## Molecular identification of causative bacterial disease in giant gourami (*Osphronemus goramy*) based on 16S rRNA gene from extensive fishpond in Banjarnegara Regency, Indonesia

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### ABSTRACT

The outbreak of bacterial disease often occurs in giant gourami (*Osphronemus goramy*) fish cultures. Banjarnegara Regency is the center of production of giant gourami (*O. goramy*) in Central Java, Indonesia. Thus, this research aimed to address the causative bacterial agent in giant gourami, based on 16S rRNA gene sequences. Twelve moribund fishes were collected from traditional fishponds of Banjarnegara Regency for bacterial isolation, and 440 juveniles were used to conduct Postulat Koch. Results showed that 21 bacterial isolates (SJB 01 – SJB 21) were isolated from kidney and external wounds of fishes from both GSP and TSA media. Postulate Koch assay indicated that four isolates (SJB 02, SJB 03, SJB 12, and SJB 14) led to fish mortality up to 60%, whereas illness was about 20% – 80%. Therefore, these isolates were potentially the causative agent of bacterial diseases in giant gourami. Based on the analysis of the 16S rRNA gene, the isolate SJB 02, SJB 03, SJB 12, and SJB 14 were closely related to *Aeromonas hydrophilla* (97.55%), *Aeromonas veronii* (98.92%), *Pseudomonas otitidis* (98.45%), and *Aeromonas jandaei* (97.74%), respectively.

### INTRODUCTION

The giant gourami (*Osphronemus goramy*) is one of Indonesian indigenous fish species representing an export commodity that is widely distributed in the Southeast Asian countries and the Chinese region (Centre for Marine and Fisheries Extension and Community Development, 2011). In the market, this important freshwater herbivorous fish has a relatively high price of around 3.2 – 3.9 USD/kg (Sarjito *et al.*, 2020). Therefore, *O. goramy* is a seeded aquaculture commodity; it has become a prominent species not only in the ornamental field but also in the edible aquaculture industries (Azrita & Hafrizal, 2015).

In order to gain the benefit of fish production, intensive and extensive cultivation technology are implemented to increase the harvest. However, these technologies can induce fish disease as a result of the interactions between the host, the pathogen, and the environment (**Kusumawardhani *et al.*, 2015; Rozi *et al.* 2018**). The causing agent of most diseases in fish is the pathogenic bacteria, especially the Gram-negative bacteria, such as *Aeromonas* sp., *Vibrio* sp., *Pseudomonas* sp., *Edwardsiella* sp., and *Flexibacter* sp. (**Uno *et al.*, 2015; Koulenti *et al.*, 2019**). The high abundance of these bacteria is generally found in the annual transition of the monsoon season between July and August.

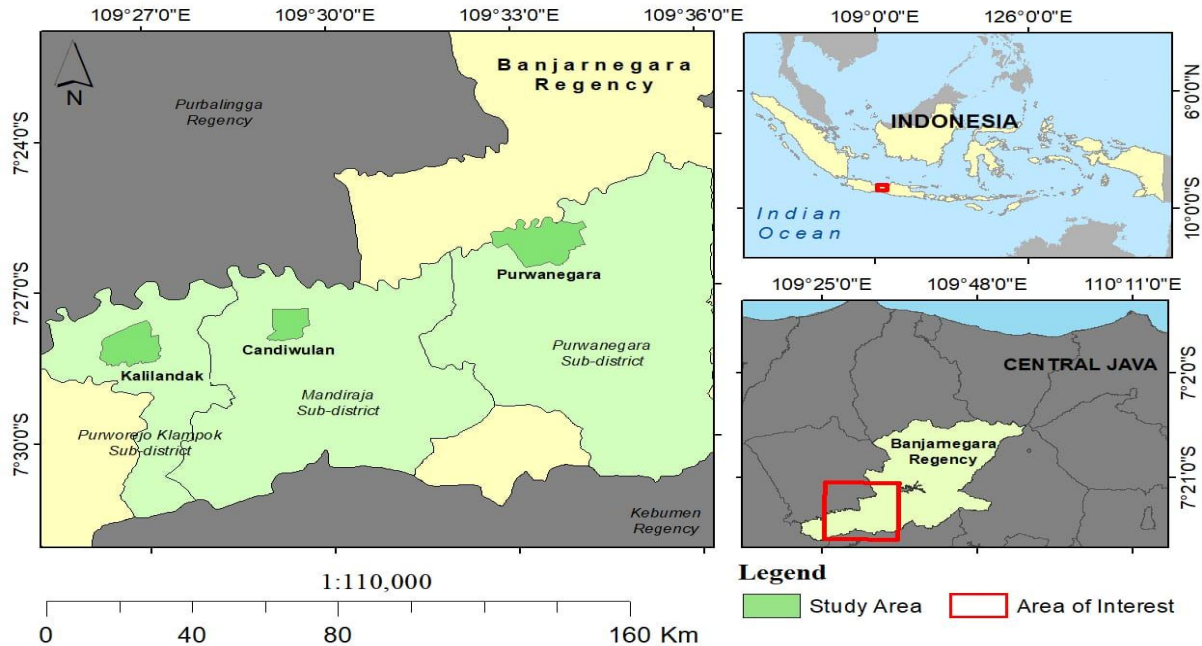
Bacterial diseases are still forming major problems in gourami culture. The clinical signs of bacterial diseases in the gourami were paleness and the skin blackness; hemorrhage around the mouth, fins and tail, in addition to exophthalmia, fin root, body wounds, and darkened liver and kidneys (**Jagoda *et al.*, 2014**). Pathogens including *A. hydrophila* dan *A. veronii* (**Hossain, 2008**), *Vibrio cholerae*, *V. anguillarum*, *P. fluorescens* (**Murwantoko, 2013**) *A. caviae*, *A. sobria* (**Benhamed *et al.*, 2014; Shi *et al.*, 2019**), *Mycobacterium* spp. (**Hamed *et al.*, 2018**), *Nocardia* sp. (**Chen *et al.*, 2019**) were determined as causative agents of bacterial diseases in gourami. These pathogens cause a high mortality rate in larvae, fingerlings, adults and broodstocks (**Rozi *et al.*, 2017**).

Banjarnegara Regency is one of the centers of giant gourami (*O. gouramy*) productions in Central Java, Indonesia (**Directorate General of Aquaculture, Ministry of Marine Affairs and Fisheries, 2015**). In 2018, a mass mortality was recorded in a traditional fishpond in Banjarnegara. A clinical sign accounted to accidental death were red spotting and fleck (**Hadiyanto, 2020**). However, the bacteria causative agent of this disease was yet unknown. To reveal the causative agents of bacterial diseases in gourami, this study aimed to identify the bacterial pathogen causing diseases in giant gourami, based on 16S rRNA gene from Banjarnegara Regency, Central Java, Indonesia. Postulate Koch was carried out to confirm the bacterial causative agents. Thus, the result could be performed to create a sustainable design of prevention and treatment strategy against the bacterial diseases.

## MATERIALS AND METHODS

### Sampling of Gourami and Bacterial Isolation

Gourami samples were taken from traditional fishponds in Kalilandak, Candiwulan, and Purwanegara sub districts in Banjarnegara Regency (Fig. 1). Based on the clinical status, 12 moribund fishes that were presumed to be infected by bacteria were collected. Five fishes were taken from Purwanegara; three and four fishes were collected from Candiwulan and Kalilandak, respectively.



**Fig. 1.** Study areas are shown in dark green color belonging to Kalilandak, Candiwulan, Purwanegara in Banjarnegara Regency, Central Java, Indonesia

The fishes were brought with containers to the Central Laboratory of Research and Service Diponegoro University to keep the fish alive for bacterial isolation. Glutamate starch penicillin (GSP) and tryptic soybean agar (TSA) media were used to isolate the bacteria from kidney and external wounds of moribund gourami, bacterial colonies, and then purified by re-culturing a single colony to the new plate (Madigan *et al.*, 2015).

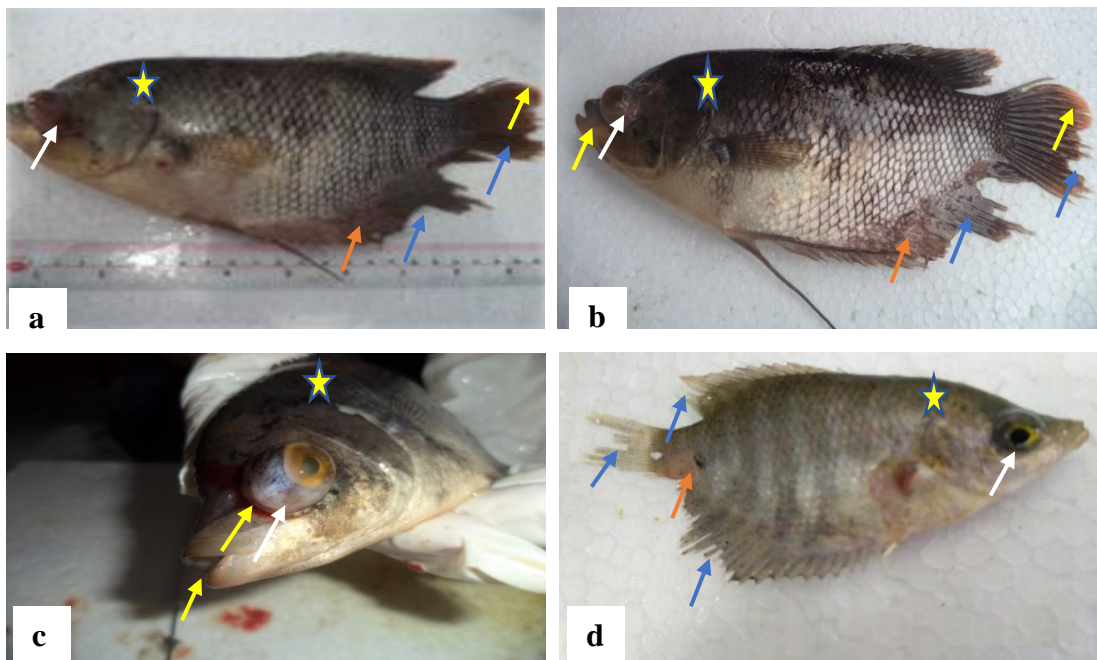
### Postulat Koch Assay and Molecular Identification

Twenty-one bacterial isolates were tested using 440 healthy gourami juveniles as experimental fish. Bacterial isolates ( $0.1 \text{ mL}$  of  $10^7 \text{ CFU/mL}$ ) were injected intramuscularly to the anaesthetised fish in low temperature ( $20^\circ\text{C}$ ). Four potential isolates of 21 isolates were identified with molecular approach based on methods previously used by Shukla *et al.* (2018). The DNA of bacterial colony was extracted using Chelex method (Giraffa *et al.*, 2000). The Polymerase Chain Reaction (PCR) was run using Eppendorf Mastercycler (Eppendorf Inc. Germany). The primer universal that used in this study was 27F ( $5'$ -AGAGTTTGATCMTGGCTCAG- $3'$ ) and 1492R ( $5'$ -TACGGYTACCTTGTTACGACTT- $3'$ ) to amplify 16S rRNA gene. The PCR products were sent for sequencing to Macrogen, Korea. The nucleotide sequence was aligned using BLAST program with the database from NCBI (Sarjito *et al.*, 2018). Further, the phylogenetic tree was constructed with MEGA X program (Sarjito *et al.*, 2017; Sarjito *et al.*, 2018).

## RESULTS AND DISCUSSION

### Clinical symptoms of Moribund Gourami

Clinical morphological symptoms gourami which were infected by bacterial diseases were wounded and pale of the body, hemorrhagic in some parts of the body specifically in the mouth, tail, and fins root area, and discoloration of kidney and liver organs that turned darker. These morphological symptoms (**Figs. 2a,b**) and the behaviour abnormalities such as decreased appetite, lethargy, and swimming imbalance were also observed in tested gourami. The clinical signs reported above also have been reported by **Benhamed *et al.*, (2015)**; **Rozi *et al.*, (2018)**; **Sun *et al.*, (2018)**; **Sarjito *et al.*, (2020)** .



**Fig. 2.** Clinical symptoms in the fish samples (a and b) and tested fish (c and d) : exophthalmia (white arrows); fins root area (blue arrow); haemorrhage (yellow arrow); wound (brown arrow); and pale of the body (star).

### Bacterial isolates of Moribund Gourami

Twenty-one bacterial isolates were isolated from kidney and external wounds of moribund gourami (**Table 1**). The Postulate Koch assay showed that four isolates, namely SJB 02, SJB 03, SJB 12, and SJB 14 were able to cause disease symptoms in range 20% up to 80% and mortality 0 % up to 60% (**Table 2**). The clinical sign between the tested Postulat Koch fish and the sampled fish from traditional fishpond were similar (**Fig. 2 c,d**).

**Table 1.** Characteristic of Isolates Bacteria Associated with Gourami from Banjarnegara, Central Java, Indonesia

No	Isolate Code	Media	Source	Colony		
				Colour	Form	Characteristic
1	SJB 01	TSA	Wound	White	Circular	Convex
2	SJB 02	GSP	Wound	Yellow	Circular	Convex
3	SJB 03	GSP	Kidney	Yellow	Circular	Convex
4	SJB 04	TSA	Wound	White	Circular	Umbonate
5	SJB 05	GSP	Wound	Yellow	Circular	Convex
6	SJB 06	GSP	Wound	Red	Circular	Convex
7	SJB 07	TSA	Kidney	White	Circular	Convex
8	SJB 08	TSA	Kidney	White	Circular	Flat
9	SJB 09	TSA	Wound	Yellow	Circular	Convex
10	SJB 10	TSA	Wound	White	Circular	Convex
11	SJB 11	GSP	Kidney	Red	Circular	Convex
12	SJB 12	TSA	Wound	White	Filamentous	Flat
13	SJB 13	TSA	Kidney	White	Circular	Convex
14	SJB 14	TSA	Kidney	White	Circular	Convex
15	SJB 15	TSA	Wound	White	Circular	Convex
16	SJB 16	GSP	Wound	Red	Circular	Convex
17	SJB 17	GSP	Wound	Yellow	Circular	Convex
18	SJB 18	GSP	Wound	white	Circular	Convex
19	SJB 19	TSA	Kidney	White	Filamentous	Flat
20	SJB 20	TSA	Wound	White	Circular	Convex
21	SJB 21	TSA	Wound	white	Circular	Convex

**Table 2.** Four Selected Bacteria Suspected as Causative Agent Associated with Bacterial Diseases from Banjarnegara Regency

Isolate Code	Sick Gourami (%)	Total Mortality of Gourami (%)
SJB 02	20	0
SJB 03	20	0
SJB 12	80	0
SJB 14	60	60

The present study also showed fishes that injected by 18 others isolates and PBS (Phosphate Buffered Saline) solution had a 100% survival rate and normal behaviour. Therefore, few samples positively confirmed as causative agents associated with bacterial diseases in gourami from Banjarnegara. Based on 16S rRNA sequence analysis using Blast System (**Table 3**), the results showed that causative agents of bacterial diseases on giant gourami from traditional pond of Banjarnegara, i.e.: SJB 02, SJB 03, SJB 12, and SJB 14 were closely related to *Aeromonas hydrophila* (97.55%), *Aeromonas veronii*

(98.92%), *Pseudomonas otitidis* (98.45%), and *Aeromonas jandaei* (97.74%) respectively.

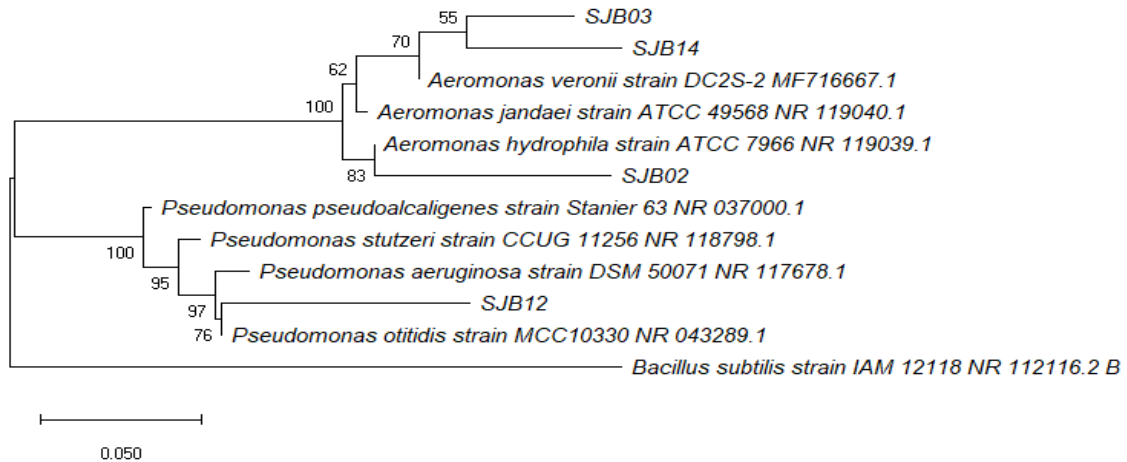
**Table 3.** Analysis of Four Selected Isolates Compared with BLAST System

Isolates	Close Relative	Homology (%)	Acc. Number
SJB02	<i>Aeromonas hydrophila</i> strain ATCC 7966	97.55	NR_119039.1
SJB03	<i>Aeromonas veronii</i> strain ATCC 35624	98.92	NR_119045.1
SJB12	<i>Pseudomonas otitidis</i> strain MCC10330	98.45	NR_043289.1
SJB14	<i>Aeromonas jandaei</i> strain ATCC 49568	97.74	NR_119040.1

**Table 3** mentioned that from four isolates, three of them are including to genus *Aeromonas*, this bacteria is a Gram-negative bacteria commonly isolated from environmental, clinical, and drinking water samples (Grilo *et al.*, 2020). *Aeromonas* sp. bacteria have been isolated from imported gourami *Colisa lalia* in Korea (Kusumawaty *et al.*, 2017). *Aeromonas* spp. is responsible for haemorrhagic septicaemia, a disease affecting a wide variety of freshwater and marine fish (Zhang *et al.*, 2020). *Aeromonas hydrophila* is the causative agent of MAS (Motile *Aeromonas* Septicemia) in both farmed and wild fishes (Nahar *et al.*, 2016). The study from Rozi *et al.* (2018) showed that the death rate of 90 – 100% in a short time was caused by *A. hydrophila* and commonly reported as a bacterial pathogen in gourami, common carp *Cyprinus carpio* (Yonar *et al.*, 2020), tilapia *Oreochromis niloticus* (Oliveira *et al.*, 2013; Sarjito *et al.*, 2021), cat fish *Clarias garipenaeus* (Sarjito *et al.*, 2018), goldfish *Carassius auratus*, snakehead *Ophicephalus striatus*, mullet *Mugil cephalus*, goby *Glossogobius guirus*, and pangasius *Pangasianodon hypophthalmus* (Nahar *et al.*, 2016).

*Aeromonas veronii* was reported as an important fish and human pathogen (Huang *et al.*, 2020). Also, it is regarded as bacterial pathogen associated with motile *Aeromonas septicemia* in African catfish *C. garipeneus* (Anyanwu *et al.*, 2015; Sarjito *et al.*, 2018) and an agent of the bacterial hemorrhagic septicaemia (Kusumawaty *et al.*, 2017). *A. veronii* which could kill 100% of experimental fish in 24 hours (Dong *et al.*, 2017), has been isolated from *Ictalurus punctatus* (Nawaz *et al.*, 2010), giant gourami *Osphronemus gouramy* (Rozi *et al.*, 2018), *Cyprinus carpio* (Gong *et al.*, 2010), *Clarias batrachus* (Sharma *et al.*, 2017), Nile tilapia *Oreochromis niloticus* (Yilmaz 2019; Raj *et al.*, 2019), and *Astronotus ocellatus* (Caldas *et al.*, 2019; Hoai *et al.* 2019). In high dose ( $8,9 \times 10^6$  CFU/fish) *A. veronii* and ( $3.7 \times 10^6$  CFU/fish) *A. jandaei* could kill 100% of experimental fish in 24 hours (Dong *et al.*, 2017). Moreover, *A. jandaei* cause mass mortality of *O. niloticus* culture in Brazil (Assane *et al.*, 2021).

The other species is *Pseudomonas otitidis*, this species has been related to aquatic infections in humans and also can be found as a free-living in some environments (Tan *et al.*, 2015; Peix *et al.*, 2018). *P. otitidis* is closely relationship to *P. aeruginosa* from genotypically and phenotypically and this similarity likely accounts for the related identification of this new pathogenic species within the *Pseudomonas* genus (Kacaniova *et al.*, 2019). *P. otitidis* was found in wastewater in Saudi Arabia (Ibrahim and Elsalam, 2018) and also in wastewater treatment plant (Mehri *et al.*, 2011). In addition, *P. otitidis* had been isolated from freshwater ornamental fish species imported into Oregon from Florida, Singapore, and Colombia (Kim *et al.*, 2016). The present research proved that bacterial causing diseases in gourami was related with Genus *Aeromonas* and *Pseudomonas*. The phylogenetic of the bacteria associated with bacterial diseases on gourami from Banjarnegara regency (Fig. 3) showed the relationship between query strain in the present study and other related members of the genus *Aeromonas* and *Pseudomonas*.



**Fig. 3.** Phylogenetic of the Bacteria Associated with Bacterial Diseases on Gourami from Banjarnegara Regency Constructed with Maximum-Likelihood Analysis in 1000 Replicates of Bootstraps Value.

Even though postulate result showed only *A. jandaei* (SJB14) causing 60% mortality of fish test, but this consortium of causative bacterial severely damaged the infected fish. Therefore, further research should be addressed to the quorum sensing between these causative agents, which lead to obtrusive fatality of the giant gourami.

## CONCLUSION

Based on molecular identification of isolated causative bacterial diseases in giant gourami in Banjarnegara Regency, the four isolates were *Aeromonas hydrophilla* (SJB02), *Aeromonas veronii* (SJB03), *P. otidis* (SJB12), and *A. jandaei* (SJB14).

According to Postulate Koch assay, *A. jandaei* was considered to be the most pathogenic bacteria that led to the death of gouramy until 60%.

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