Antibacterial potential of bacteria isolates from *Hypostomus plecostomus* fish intestine against fish pathogens

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Abstract. Diseases caused by bacteria have become are a serious problem in fish ecosystems leading to a significant number of deaths. Bacteria that often infect the fish are *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Edwardsiella ictaluri*, *Pseudomonas aeruginosa*, and *Vibrio alginolyticus*. Antibiotics are generally used to cure bacterial diseases; however uncontrolled use can lead to bacteria developing resistance. One way to combat the resistance is by looking for new compounds from microbes. The objective of this research is to identify the type of bacterium from *Hypostomus plecostomus* intestine that can inhibit the growth of pathogen bacteria such as *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Edwardsiella ictaluri*, *Pseudomonas aeruginosa*, and *Vibrio alginolyticus*. This research used an explorative method and experiment. Bacteria were isolated from the intestine of *Hypostomus plecostomus* fish. It was cultured on tryptic soy agar (TSA) medium and purified. The isolates from the purified breed went through antagonistic test by diffusion method against *Aeromonas hydrophila*, *Aeromonas salmonicida, Edwardsiella ictaluri*, *Pseudomonas aeruginosa* and *Vibrio alginolyticus* pathogen bacteria. The bacterium isolates from the fish intestine were identified by using the 16S rDNA. The antagonistic test results from the *Hypostomus plecostomus* fish intestine with the code JS1-22, JS2-22, JS3-22 that can inhibit pathogen bacteria growth in fish were categorized as very effective. The findings from using BLAST system on isolate JS1-22 isolate showed DNA similarity with *Bacillus paramycoides* (99.59%), JS2-22 isolate with *Bacillus* sp. (99.53%), and JS3-22 isolate with *Bacillus megaterium* (99.65%).

Key Words: 16S rDNA, aquaculture, *Bacillus*, fish diseases.

Introduction. Bacterial diseases are a serious issue in fish farming as they can cause huge economical loss (Cai et al 2018; Liu et al 2017). Bacteria often found to infect fish are *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Edwardsiella ictaluri*, *Pseudomonas aeruginosa* and *Vibrio alginolyticus* (Chen et al 2019; Zhang et al 2016; Stratev & Odeyemi 2016; Li et al 2021; Kalindamar et al 2021).

Aeromonas hydrophila bacterium can cause septicaemia to fresh water fish (Chaix et al 2017; Xia et al 2017). Infection from *Aeromonas hydrophila* can damage fish gills and intestines (Abdelhamed et al 2017). *Aeromonas hydrophila* was reported to infect *Lates calcarifer* (Hamid et al 2016), *Channa striata* (Samayanpaulraj et al 2020), *Ictalurus punctatus* (Jubirt et al 2015), *Cyprinus carpio*, and *Pangasianodon hypophthalmus* fish species (Rasmussen-Ivey et al 2016).

Aeromonas salmonicida is the bacterium responsible for furunculosis, a serious disease to the salmonid fish species. This disease has a significant impact to the economic loss to the salmon fish industry globally (Li et al 2021). *Aeromonas salmonicida* bacterium is a water pathogen that can infect several types of fish (Xu et al 2021), able to cause haemorrhage, ulcerative lesion, bleeding, and death (Valderrama et al 2019).

Edwardsiella ictaluri is an intracellular facultative pathogen, gram negative bacterium, in the Enterobacteriaceae family, and can cause enteric septicemia of catfish (Kalindamar et al 2021). *Edwardsiella ictaluri* is one of the most common pathogen

bacteria for yellow catfish (*Pangasianodon hypophthalmus*) responsible for a huge economical loss at the fingerling stage (Nhu et al 2019). *Edwardsiella ictaluri* is one of the most critical fish pathogens that cause enteric septicemia to catfish (Nguyen et al 2021).

Vibrio alginolyticus bacterium is a gram-negative bacterium with opportunistic pathogen behavior towards sea animals (Zhao et al 2018). The bacterium infection can cause exophthalmos, boils, septicemia, and damage to cornea of fish (Rameshkumar et al 2017; Wang et al 2016; Luo et al 2016). *Pseudomonas aeruginosa* was reported to infect freshwater and saltwater fish (Mishra et al 2014). The bacterium infection can be seen from red spots because of internal bleeding, darker skin color, loose scales, protruding eyes, damaged fins (Altinok et al 2006), and abnormal behavior due to disrupted motor functions and the fish is unable to swim normally (Baldissera et al 2017).

Antibiotics are generally used to cure bacterial diseases; however antibiotic use has side effects to the fish and the environment (Rodriguez-Mozaz et al 2020; Cao et al 2018). Antibiotics can build up in the fish and bacteria can develop resistance (Rasul & Majumdar 2017).

Efforts to overcome the resistance are by finding new compounds from a variety of sources, one of them being microbes (Saryono et al 2015). *Bacillus* sp., *Bacillus cereus*, *Bacillus toyonensis* and *Pseudoalteromonas* sp. are able to inhibit growth of *Vibrio alginolyticus*, *Aeromonas hydrophila* and *Pseudomonas aeruginosa* pathogen bacteria (Setiaji et al 2020). Bacteria from the Bacillus genes are known to produce antimicrobial compounds such as *Bacillus* sp. (Xiu et al 2017), *Bacillus pumilus* (Zidour et al 2017), *Bacillus amyloliquefaciens* (Chakraborty et al 2017), and *Bacillus subtilis* B5 (Li et al 2016).

Hypostomus plecostomus fish is a species that can live in dirty and contaminated bodies of water (Jumawan et al 2016). Its capability to live in contaminated water shows that it has microorganisms that can inhibit pathogen bacteria growth in its digestive system. Therefore, this aspect needs to be explored further, particularly for the types of bacteria. The objective of this research is to identify the types of bacteria in the intestine of *Hypostomus plecostomus* fish which can inhibit pathogen bacteria growth in fish.

Material and Method

Isolation and bacteria antagonistic test. This research was performed from the month of August to December 2022. *Hypostomus plecostomus* fish was sourced from Sail River in Pekanbaru city (0°32’37”N, 101°28’09”E). Bacteria isolate from *Hypostomus plecostomus* fish intestine was spread on a tryptic soy agar (TSA) media and incubated at 30°C for 24 hours. Bacteria isolates from *Hypostomus plecostomus* fish intestine was antagonistic tested on *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Edwardsiella ictaluri*, *Pseudomonas aeruginosa* and *Vibrio alginolyticus* pathogen bacteria by the diffusion method. 30 µl of pathogen bacteria were tested, planted in TSA nutrient medium and spread evenly with L rod. After the pathogen bacteria breed filled medium solidified, Oxytetracycline antibiotic paper was placed as the positive control and 30 µl of bacteria isolate from *Hypostomus plecostomus* fish intestine was dropped onto the paper disc. Then it was incubated at 30°C for 24 hours. The size of antagonistic activity was measured by measuring the diameter of clear zone around the disc.

Bacteria identification using 16S rDNA method. Molecular identification of the bacteria was done by using 16S rDNA (Feliatra et al 2016). DNA from *Hypostomus plecostomus* fish intestine was isolated. Electrophoresis was performed to find the total DNA of the bacteria. Once the DNA bands were produced, PCR process was done to replicate the DNA bands up to ±1500 bp. The bacteria’s DNA sample was sent to PT. Genetika Science Indonesia for sequencing. Further identification on the bacteria species was done using tools from the website of the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/guide/dna-rna) (Sayers et al 2022).

Statistical analysis. Data extracted from antagonistic test and bacteria identification using 16S rDNA method was descriptively analyzed and compared with the relevant literatures.

Results and Discussion

Antagonistic test toward fish pathogen bacteria. Antagonistic tests were done to find if the bacteria from *Hypostomus plecostomus* fish intestine can fight against pathogen bacteria. The isolation results of the bacteria from *Hypostomus plecostomus* fish intestine produced 3 isolates with codes given to be JS1-22, JS2-22, and JS3-22. These three isolates were able to inhibit the growth of *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Edwardsiella ictaluri, Pseudomonas aeruginosa*, and *Vibrio alginolyticus* pathogen bacteria. This was shown by the presence of inhibition zone that formed around the paper disc. The size of the inhibition zone produced from *Hypostomus plecostomus* fish intestine bacteria ranges from 8.4 mm to 17.5 mm (Table 1).

Table 1

Antagonistic test of *Hypostomus plecostomus* fish intestine bacteria against pathogen bacteria

Pathogen bacteria	*Inhibition zone diameter (mm)*		
	Isolate JS1-22	*Isolate JS2-22*	*Isolate JS3-22*
Aeromonas hydrophila	10.4±0.07	14.9±0.07	16.1±0.07
Aeromonas salmonicida	8.4±0.00	8.5±0.00	8.7±0.00
Edwardsiella ictaluri	8.6±0.07	9.6±0.00	10.2±0.07
Pseudomonas aeruginosa	11.2±0.07	10.9±0.07	14.5±0.07
Vibrio alginolyticus	10.5±0.00	10.2±0.00	17.5±0.00

Based on the antagonistic test, it shows that the bigger the transparent zone produced on tests in vitro, the stronger the inhibition of a microbe. Generally, the potential of bacterial growth inhibition is caused by the secondary metabolite produced by microbes like antibiotics, bacteriocin, siderophores, lysosomes, protease, hydrogen peroxide, or altering the media's pH level by producing certain organic acids. Inhibition zones formed on the culture media were caused by inhibition by antimicrobial compound towards microbial cells. Antimicrobial compound mechanisms can occur by disrupting or damaging cell wall constituents, reacting with cell membranes, increasing cellular permeability, essential enzymes inactivation, destruction or inactivation function and genetic materials (Sari et al 2013).

Bacteria isolates from *Hypostomus plecostomus* fish intestine (JS1-22, JS2-22, JS3-22) inhibition capability towards *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Vibrio alginolyticus* bacterial growth can be categorized as strong. However, bacteria isolates from *Hypostomus plecostomus* fish intestine (JS1-22, JS2-22, JS3-22) inhibition capability towards *Aeromonas salmonicida* and *Edwardsiella ictaluri* bacterial growth can be categorized as medium.

Antagonistic test of bacteria isolates from *Hypostomus plecostomus* fish intestine on pathogen bacteria showed different results to each isolate. Different diameter of inhibition zones produced by the bacteria isolates from *Hypostomus plecostomus* fish intestine were caused by the different capabilities of producing antibacterial materials. The inhibition capability of bacteria isolates from *Hypostomus plecostomus* fish intestine are the highest against other pathogen bacteria. The difference in diameter of the inhibition zone was also caused by the resisting capability of the pathogen bacteria against bacteria isolates from *Hypostomus plecostomus* fish intestine. The antagonistic test results showed that bacteria isolates from *Hypostomus plecostomus* fish intestine were able to damage and degrade the structure of pathogen bacteria cell wall resulting in pathogen bacteria growth inhibition.

The growth inhibition capability of an antibacterial compound depends on the concentration of antimicrobial content and the type of antimicrobe produced. The higher the concentration of an antimicrobe, the larger the transparent zone formed. This is due

to higher antimicrobial content, meaning more active substances are present thus increasing the effectivity in inhibiting the bacterial growth, which determined a larger inhibition zone (Rastina et al 2015; Rahmah et al 2017).

The inhibition zone formed on the pathogen bacteria culture media indicated that secondary metabolite of bacteria isolates from *Hypostomus plecostomus* fish intestine contain antimicrobe like antibiotic, bacteriocin, or other compounds. Microbe populations were able to release chemical substances with bactericidal or bacteriostatic capabilities that can affect other bacteria (Verschuere et al 2000). Antimicrobial compound biosynthesis has an important act during the bonding process of target colonization resulting in competition for space and nutrition amongst the microbes (Romanenko et al 2008).

Sequence analysis and BLAST DNA of bacteria from Hypostomus plecostomus fish intestine. The results of DNA extraction of bacteria isolates from *Hypostomus plecostomus* fish intestine (JS1-22, JS2-22, JS3-22) were analyzed with PCR by using primer 24F:5'-AGAGTTTGATCCTGGCT-3' and 1541R: 5'-AAGGAGGTGATCCAGCC GCA-3'. The electrophoresis result of bacteria isolates from *Hypostomus plecostomus* fish intestine and the PCR obtained were in the form of bands (Figure 1). This indicated that the primer used succeeded in amplifying specific DNA fragments on the bacteria isolates from *Hypostomus plecostomus* fish intestine. 16S rDNA amplification became a standard to study phylogeny and the variety of saltwater microorganisms. 16S rDNA method is specifically used to identify bacteria (Benga et al 2014). Identification with 16S rDNA method was used to determine distribution of the bacterial species (Wang et al 2014).

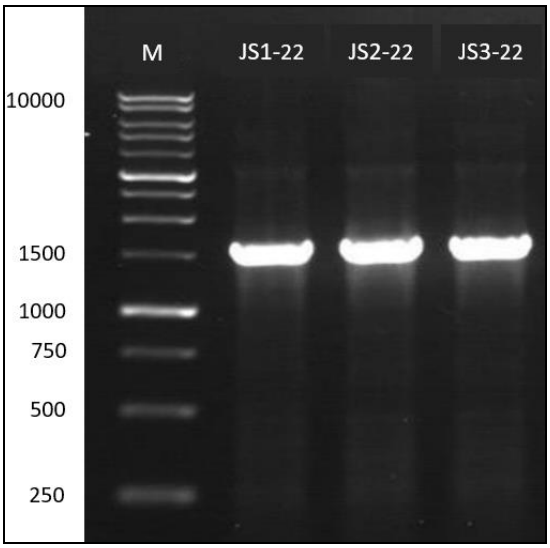

Figure 1. DNA electrophoresis result of bacteria isolates from *Hypostomus plecostomus* fish intestine.

The identification result of bacteria isolates from *Hypostomus plecostomus* fish intestine can be seen based on the highest homology in the BLAST system results. The findings from BLAST showed that JS1-22 isolate is the closest to *Bacillus paramycoides*, JS2-22 isolate with *Bacillus* sp., and JS3-22 with *Bacillus megaterium*. Furthermore, the genetic relationship between isolates can be seen in the phylogenetic tree (Figure 2).

The homology level of bacteria isolates ranges from 99.53% to 99.65% with the type of bacteria available in GenBank database (Sayers et al 2022) (Table 2). The validation of species level can be defined with the similar order 16S rDNA 99%, and 97% which can be determined as genus level. Similarity of below 97% can be considered not compatible for genus identification (Cai et al 2003). If the level of homology is lower than 93%, the bacteria isolates were considered to be of new species, as the base nitrogen level has not been recorded in GenBank database (Hagstrom et al 2000).

Table 2
Sequencing findings of 16S rDNA of bacteria isolates from *Hypostomus plecostomus* fish intestine using BLAST system

Isolate	*Species*	*Strain*	*Access code*	*Query coverage (%)*	*Homology (%)*
JS1-22	*Bacillus paramycoides*	MCCC	NR157734.1	100	99.59
JS2-22	*Bacillus* sp.	IC-1C2	MT649293.1	99	99.53
JS3-22	*Bacillus megaterium*	MP-5	DQ462195.1	100	99.65

Bacillus paramycoides is a bacterium potentially useful for degrading acephate and remediation of contaminated soil (Ren et al 2020). The results showed that *Bacillus paramycoides* SP3 can be exploited to get rid of environmentally friendly selenite from contaminated locations through biosynthesis (Borah et al 2021). *Bacillus paramycoides* bacterium isolated from mulch waste recycling plant showed extraordinary capability to degrade polyethylene (Wu et al 2022).

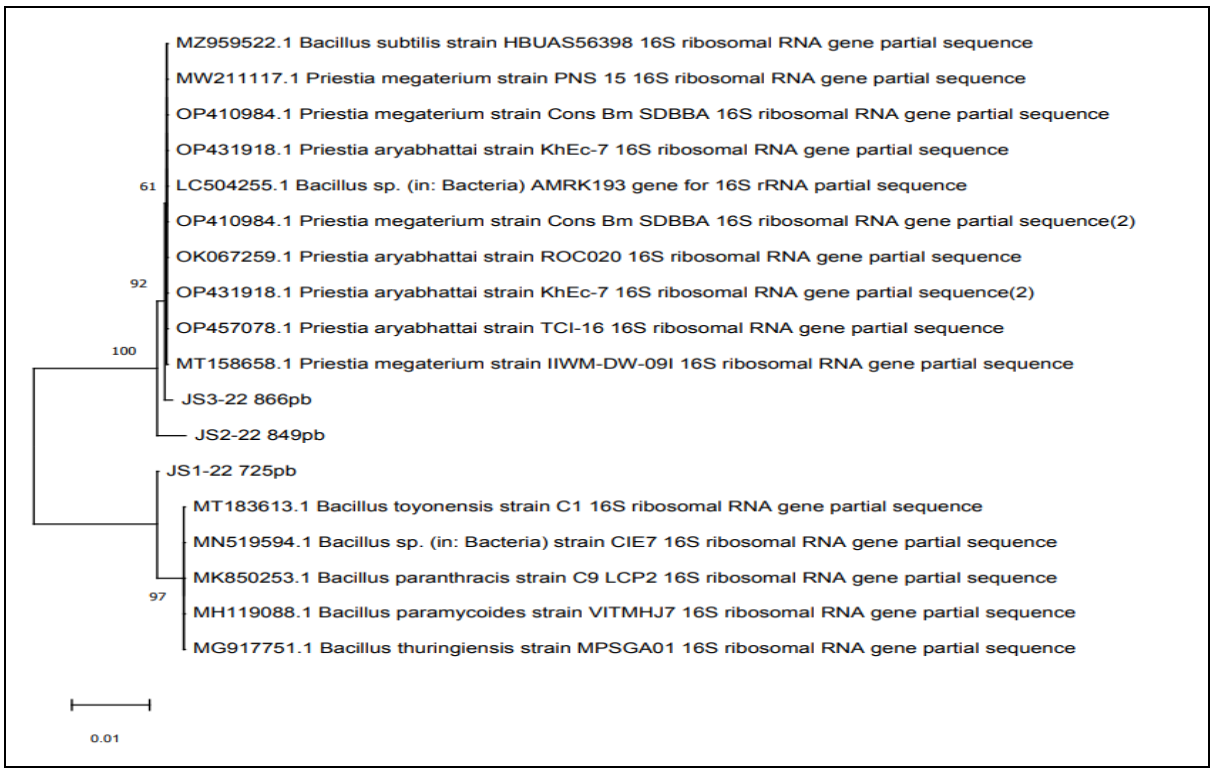

Figure 2. Phylogenetic tree bacteria isolates from *Hypostomus plecostomus* fish intestine (generated using MEGA X software).

Bacteria from the *Bacillus* groups are often used in research related to probiotics, as they can produce antimicrobial microbes. *Bacillus* sp. bacterium has the capability to produce antibiotics, significant in the process of nitrification and denitrification. It can mineralize complex organic matter in the form of polysaccharide, protein, and cellulose. As an individual species, *Bacillus* sp. has the capability to be a probiotic, therefore it was utilized in fish culture (Feliatra et al 2018). Probiotic bacteria are able to produce antibacterial compounds that can suppress the growth of pathogen microbes (Effendi 2017). The *Bacillus* species produced at least 66 types of antibiotics and certain strains from the *Bacillus* species are biocontrol agents (Nishijima et al 2005). Secondary metabolite of *Bacillus pumilus* bacterium that originated from the sea can produce ammonium salt as an antimicrobial compound (Zidour et al 2017). *Bacillus* sp. from the sea has a structure similar to Pumilacidin that is effective to suppress motility of *Vibrio alginolyticus* bacterium (Xiu et al 2017).

Bacillus megaterium NCT-2 has the potential application to remediate soil (Chi et al 2020). It can form a protection layer on metal and reduce biofilm adsorption because it

has antimicrobial properties (Kokilaramani et al 2020). *Bacillus megaterium* can produce polyhydroxyalkanoates (PHAs) using volatile fatty acid from the fermentation of acidogenic food waste (Vu et al 2021). *Bacillus megaterium* strain JX285 that was isolated from rhizophore red soil sample can dissolve inorganic phosphor, increasing the phosphor count that is available and improving plant growth (Huang et al 2019). Purification, structural characterization, and antioxidant activity from *Bacillus megaterium* PFY-147 has implications in the field of food, medicine, and pharmacy industries (Pei et al 2020).

Conclusions. The research obtained three bacteria isolates from *Hypostomus plecostomus* fish intestine coded JS1-22, JS2-22, JS3-22 that can inhibit the growth of pathogen bacteria in fish, in the effective category. The results from using BLAST system found that JS1-22 isolate is the closest to *Bacillus paramycoides* (99.59%), JS2-22 isolate with *Bacillus* sp. (99.53%), and JS3-22 with *Bacillus megaterium* (99.65%).

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Conflict of interest. The authors declare that there is no conflict of interest.

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