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Antibiotic Resistance in *Vibrio* spp. Causing Disease in Barramundi Fish (*Lates calcarifer*) Eye

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Abstract

The cultivation of barramundi fish (*Lates calcarifer*) is widely carried out because barramundi fish has a relatively high economic value. The disease in question is caused by *Vibrio* spp. bacteria, which are marine bacteria that often affect barramundi fish, and the treatment for the bacteria is antibiotics. Therefore, research was conducted to test several types of antibiotics and observe the bacterial resistance reaction to the administration of these antibiotics. The methods used in this study included media preparation, purification, and microscopic characterization, liquid media preparation, solid media preparation, and antibiotic sensitivity testing. *Vibrio* spp. are still sensitive to antibiotics at a rate of 61.27%, resistant at 18.14%, and intermediate at 20.59%. Bacterial isolate characteristics were divided into several types: Irregular, Punctiform, Round, and Round Umbonate. Irregular, Punctiform, Round, and Round Umbonate are still sensitive to antibiotics at rates ranging from 37.5% to 66.67%. Other results include intermediate and resistance with results ranging from 15.63% to 37.5% and 15.10% to 25.00%, respectively. The highest sensitivity results were found in antibiotics such as Gentamycin (98.94%), Co-Amoxiclave (86%), Tetracycline HCl (72.55%), Ciprofloxacin HCl (82.35%), Azithromycin (52.94%), and Doxycycline (54.90%). On the other hand, the highest intermediate and resistance results were found in chloramphenicol (52.94%) and Ampicillin (60.78%), respectively. Ampicillin is considered to be the antibiotic that the bacteria are resistant to because of its small inhibitory zone.

1. Introduction

Fish farming is a commonly practiced activity among the people in Indonesia. This is because Indonesia is a maritime country with a large part of its territory consisting of water bodies. This makes the country rich in its aquatic natural resources. One of the commonly practiced forms of aquaculture is marine fish farming, including white barramundi fish. These fish can grow rapidly and have high economic value, with a fairly extensive export market, making them suitable for cultivation (Rayes *et al.*, 2013). However, one of the challenges in fish farming is the presence of contagious diseases caused by pathogenic bacteria. These bacteria can be inhibited in their growth by using antibiotics (Talpur, 2014).

Antibiotics are medications commonly used in the world of aquaculture to control and treat diseases in barramundi fish caused by pathogenic bacteria. However, excessive antibiotic use can be harmful to the environment. This is because residues of these chemicals and antibiotics can accumulate in the soil/sediment (Öntaş *et al.*, 2016). Therefore, the use of antibiotics needs to be carefully considered. Besides being harmful to the environment, the bacteria that affect the fish will develop resistance to these antibiotics (Moyo *et al.*, 2011). Resistance itself is a negative consequence of inappropriate antibiotic use, unclear usage methods, and excessive drug use (Mahmudah *et al.*, 2016).

Vibrio spp. bacteria are opportunistic bacteria that can be found in brackish water, estuaries, and the sea. These bacteria thrive in a marine environment with a salinity of 20-40 ppt. *Vibrio* spp. live in symbiosis with both marine and freshwater biota and are halophilic, capable of producing proteolytic and chitinolytic enzymes (Ihsan and Retnaningrum, 2017). These bacteria can cause diseases in barramundi fish (*Lates calcarifer*) by attacking various parts of their bodies, including their eyes, which can be detrimental to barramundi fish farming as it can lead to mortality. This

has led marine science students to conduct research on antibiotics that can inhibit the growth of bacteria causing eye diseases in barramundi fish in a cultivation setting. In addition to determining the types of antibiotics needed, this research also tests bacterial resistance to antibiotics.

2. Material and methods

2.1 Preparation of *Vibrio* sp. slant agar.

Nutrient Broth (Merck) was weighed at 1.3 grams, and agar (Merck) was weighed at 1.5 grams. Aquades (distilled water) at 100 ml. The weighed ingredients were placed in an Erlenmeyer flask and homogenized using a hot plate stirrer and a magnetic stirrer. After homogenization, the flask containing the medium was sealed with cotton, covered with aluminum foil, and secured with a rubber band. Then, the medium was sterilized using an autoclave for 15 minutes (after the autoclave released steam). After 15 minutes, the autoclave was turned off, and the flask containing the medium was removed. The medium was cooled to room temperature until it felt sufficiently warm, and then it was poured into 5 ml test tubes. The transfer of the medium from the Erlenmeyer flask to the test tubes had to be done aseptically, meaning it was done near a Bunsen burner as a source of flame. This was done to prevent contamination of the medium. The test tubes containing the medium were sealed with cotton and tilted to create slanted agar. The agar in the test tubes was allowed to cool and was ready for use in *Vibrio* bacterial purification (Yudiati *et al.*, 2021b).

2.2 Purification of *Vibrio* spp. isolates

Vibrio samples (from the eyes of *L. calcarifer*) previously cultured on solid media were first macroscopically identified based on colony characteristics, including shape, color, margin, and elevation. This identification was carried out to determine the bacterial colony forms, and subsequent resistance tests were performed on each identified form. After identification, *Vibrio* was purified onto slant agar media in test tubes. *Vibrio* bacteria from the Petri dish were collected using a loop with a sterile round needle that had been sterilized with alcohol and a Bunsen burner. The loop was sterilized until its tip glowed bright red, and not just the tip of the needle but also the portion of the loop closest to the needle was heated. After turning bright red, the loop was used to collect bacterial samples, and the cotton cap on the agar medium was opened, and the sample was streaked onto the slant agar medium in a zigzag pattern from the deepest end to the outer end. The test tube was then sealed with cotton and wrapped with a wrapper. Labels were added based on the previously identified bacterial characteristics and others. The above process was conducted using aseptic techniques. After streaking, the test tubes were placed in a location, such as a sterilized glass beaker/plastic container, and incubated at around 38°C in an incubator.

2.3 Preparation of liquid and solid *Vibrio* media.

Nutrient Broth (NB) was weighed at 1.3 grams and placed in an Erlenmeyer flask. It was then homogenized with 100 ml of aquades using a hot plate stirrer. After homogenization, the NB medium was briefly cooled and then transferred into vials, each containing 5 ml, sealed with cotton, and covered with aluminum foil. The prepared vials were then sterilized using an autoclave for 15-20 minutes after the sound of steam release was heard. After completion, the vials were removed from the autoclave and allowed to cool before being ready for use (Yudiati *et al.*, 2021a).

Agar (Merck) was weighed at 1.5 grams, and Nutrient Broth (Merck) at 1.3 grams. Then, both were homogenized in an Erlenmeyer flask with 100 ml of aquades using a hot plate stirrer. After the media was homogenized, it was poured into Petri dishes. Care should be taken not to pour too little or too much. Then, the solidified media was wrapped and placed in sterilized plastic (Yudiati *et al.*, 2022).

2.4 Antibiotic activity testing on *Vibrio* sp.

Paper disks, made from Whatman No. 3 filter paper and cut into small circles (6mm), were prepared and placed in a glass beaker, then autoclaved. The antibiotics to be stocked were ground to a fine powder using a mortar and pestle. Subsequently, the antibiotics were homogenized, covered with a vial cap, and wrapped 15 minutes after homogenization. Afterward, the stock was stored in the refrigerator. The antibiotics used were Ampicillin, Gentamycin, Doxycycline, Tetracycline, Chloramphenicol, Ciprofloxacin, Co-amoxiclav, and Azithromycin. The antibiotic concentrations were adjusted according to CLSI (Clinical and Laboratory Standards Institute) guidelines. The steps were performed using aseptic techniques. Each paper disk was spaced apart, and each received 20 microliters of the same type of antibiotic using a micropipette. Petri dishes were labeled according to the antibiotic applied. Then, the paper disks were allowed to dry and were ready for use. The paper disks were placed using sterile forceps. Subsequently, the Petri dishes were inverted in the incubator. The diameter was measured in the clear zones that formed between bacterial reactions and antibiotics using calipers. The inhibition zones were classified into three categories: sensitive, intermediate, and resistant (Bauer *et al.*, 1966; CLSI, 2010, 2018)

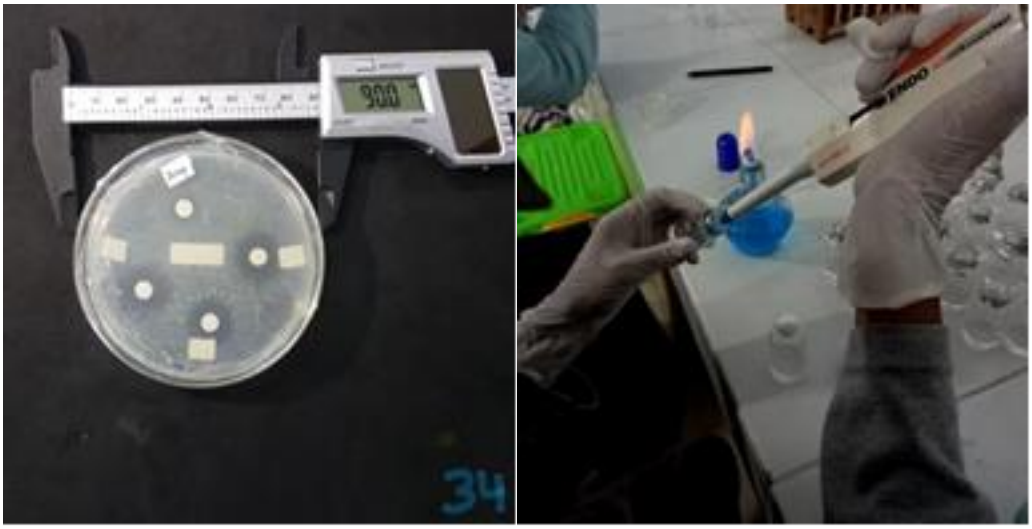

Figure 1. Antibiotic testing process

3. Results

The antibiotic testing process (Figure 1) yielded results presented in Figures 2-4.

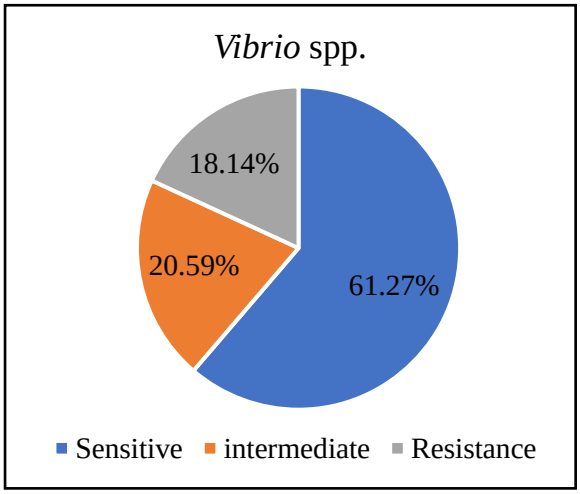

Figure 2. The total sensitivity results of antibiotics in *Vibrio* spp.

Based on Figure 2, Vibrio spp. still showed sensitivity to antibiotics at a rate of 61.27%, resistance at 18.14%, and intermediate resistance at 20.59%. The characteristics of bacterial isolates were divided into several types: Irregular, Punctiform, Round, and Round Umbonate. According to Figure 3, Irregular, Punctiform, Round, and Round Umbonate remained sensitive to antibiotics, ranging from 37.5% to 66.67%. Other results included intermediate resistance ranging from 15.63% to 37.5% and resistance ranging from 15.10% to 25.00% (Figure 3).

Figure 4 showed that the highest sensitivity results were obtained with antibiotics such as Gentamycin (98.94%), Co-Amoxiclav (86%), Tetracycline HCl (72.55%), Ciprofloxacin HCl (82.35%), Azithromycin (52.94%), and Doxycycline (54.90%). On the other hand, the highest rates of intermediate resistance were found in chloramphenicol (52.94%) and Ampicillin (60.78%), respectively.

4. Discussion

The outbreak of this disease can have economic impacts on aquaculture operations as it can reduce production, harm cultivation efforts, and even lead to mass

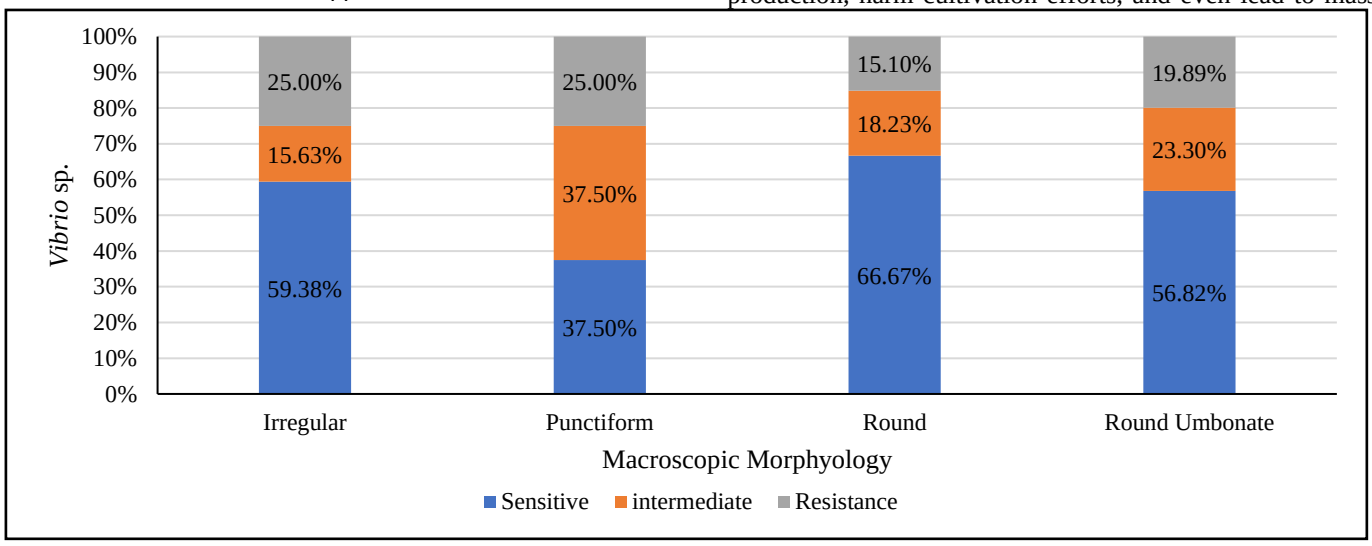

Figure 3. The results of antibiotic sensitivity testing based on the macroscopic characteristics of *Vibrio* sp.

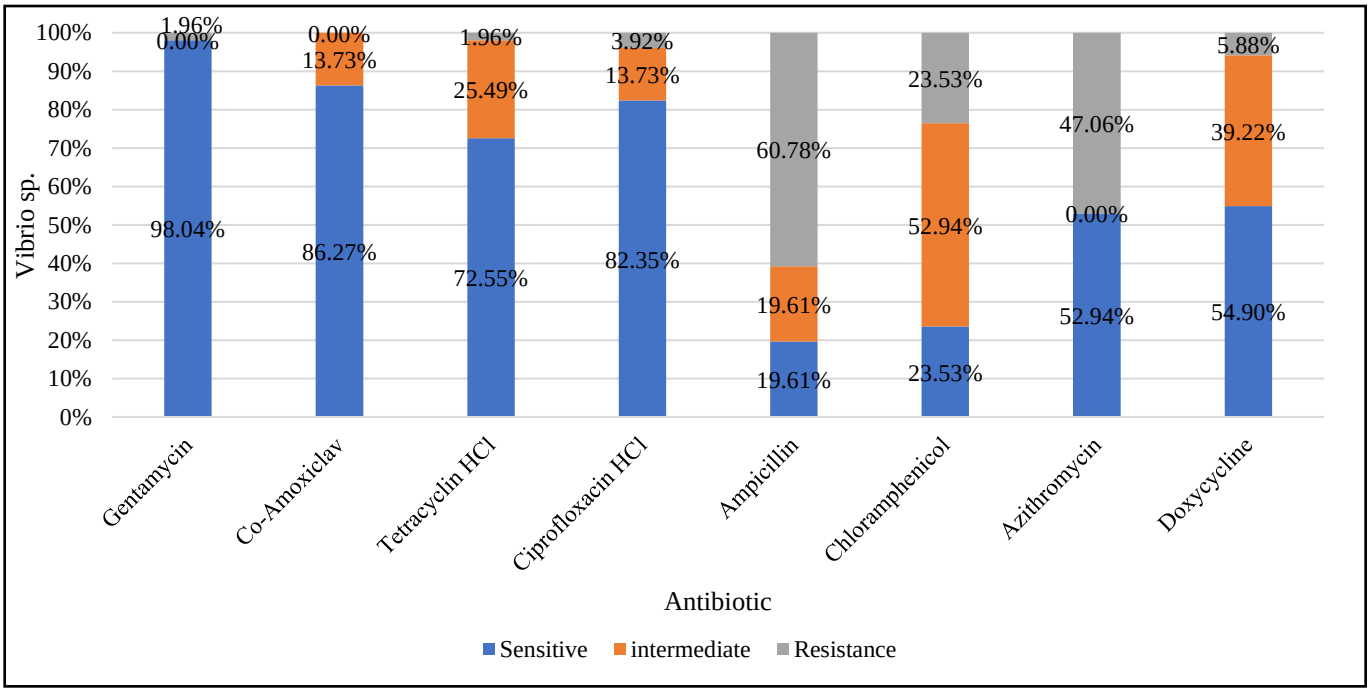

Figure 4. The results of sensitivity testing for various types of antibiotics based on *Vibrio* sp.

mortality. The disease can be caused by the presence of *Vibrio* bacteria, which are a dominant genus of bacteria in brackish and marine water environments (Novita *et al.*, 2020). *Vibrio* spp. is a common group of gram-negative bacteria with a rod-like shape. These bacteria are naturally occurring organisms in aquatic environments, estuaries, and the sea. They are considered pathogenic bacteria, capable of causing harm to other organisms. Despite their pathogenic nature, these bacteria all originate from aquatic and marine environments, favoring warm temperatures, slightly saline brackish water, and are abundant in nature. Typically, these bacteria live by associating with marine or other aquatic organisms (Baker-Austin *et al.*, 2018).

Most of these bacteria can produce specific enzymes, such as proteolytic and chitinolytic enzymes. In addition to being pathogenic, they also have a halophilic nature, which means they thrive and reside in waters with high salt content or salinity (Ihsan and Retnaningrum, 2017). The characteristics of these bacteria include colonies with a round morphology, flat elevation, convex and shiny appearance, and green-colored colonies (Susianingsih *et al.*, 2012). Antibiotics have become life-saving drugs for many living organisms worldwide, significantly reducing morbidity and mortality. Their ability to combat infections has led to a significant improvement in health. However, the irrational use of antibiotics without a doctor's prescription can lead to the development of antibiotic-resistant bacteria (Desrini, 2015). Resistance itself is a microorganism's ability to withstand a specific antibiotic or antimicrobial. Resistance can be categorized into several types, including natural resistance, resistance due to spontaneous mutations or chromosomal resistance, and extrachromosomal resistance due to the transfer of resistant genes. Extrachromosomal resistance can also be attributed to a microorganism's genetic or non-genetic mechanisms against antibiotics (Artati *et al.*, 2018).

The emergence of antibiotic resistance is a global concern, leading to increased awareness of the relationship between antibiotic resistance and their usage. Information about antibiotic use serves as an initial benchmark for detecting the rationality of its use and controlling antibiotic resistance (Pradipta *et al.*, 2012). The primary cause of antibiotic resistance is uncontrolled and irrational antibiotic use, leading to bacteria developing resistance to multiple types of antibiotics (Niasono *et al.*, 2019).

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5. Conclusions

A wider clear/inhibition zone indicates that the antibiotic can inhibit/kill the growth of bacteria. Conversely, if the clear/inhibition zone becomes smaller/narrower, a bacterium is considered resistant to the antibiotic, rendering the antibiotic ineffective. The antibiotic that is considered resistant to the bacteria is the type of ampicillin. Vibrio spp. bacteria are already resistant to ampicillin because of their small clear/inhibition zone.

Ethics approval

No need permit to *Lates calcarifer*

Data availability statement

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Credit authorship contribution statement

IShofie Rahmah Chairani Akhmad : research. Agustien Naryaningsih : Conceptualization, methodology, validation, formal analysis, investigation, resources, writing original draft preparation, writing review and editing, visualization, supervision, project administration, funding acquisition. Both authors have read and agreed to the published version of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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