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Detection of Resistant Bacteria through Molecular Identification from Traditional Ponds in Tirang Beach, Semarang, Indonesia

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ABSTRACT

The Tirang Beach area in Indonesia is a coastal area with many fish farming ponds, raising milkfish (*Chanos chanos*), saline tilapia (*Oreochromis niloticus*), and deep-bodied mojarra fish (*Gerres erythrourus*). Aquaculture activities in this area are often hampered by bacteria that infect the farmed fish. The use of antibiotics to prevent disease is a growing trend; however, it can lead to antibiotic resistance and residues in the environment. Antibiotic resistance is a global problem that is important to research. This study aimed to monitor the existence of resistant bacteria in sediment and fish from a traditional pond as well as to identify the most resistant bacteria using a molecular approach. The research was carried out from January to April 2024, with data collection using a random sampling method. Samples were taken from the pond sediment and fish gills at three different points. Based on the results, the abundance of bacteria in the sediment and fish gills from the traditional ponds in the Tirang Beach area ranged from 1.53×10^6 to 2.45×10^7 CFU/mL and 7.2×10^5 to 5.52×10^6 CFU/mL, respectively. Furthermore, the resistance level of bacteria against Tetracycline (30 µg), Chloramphenicol (30 µg), Erythromycin (15 µg), and Ciprofloxacin (5 µg) was 36.67, 6.67, 30.00, and 56.67 percent, respectively. Therefore, the antibiotic resistance tests showed that the highest resistance was to Ciprofloxacin. Even though the abundance of bacteria in the sediment was higher than in the fish gills, the resistance bacteria in the fish gills was 82% greater than in the sediment. Moreover, observation through the 16S rRNA gene revealed that the most resistant fish bacteria was *Vibrio alginolyticus*, which is a rod-shaped Gram-negative bacterium; the most resistant bacteria in the sediment was identified as *Vibrio parahaemolyticus*, a harmful bacterium well-known as a causative agent of Acute Hepatopancreatic Necrosis Disease (AHPND). Hence, the residual antibiotic in the environment could trigger resistance in *Vibrio* spp. associated with fish and sediment in the traditional pond, despite the absence of antibiotics usage in this farming.

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1. Introduction

Tirang Beach is a tourist destination located in the Tambakrejo Village, Tugurejo, Tugu, Semarang City. Tirang Beach is strategically located close to the busy city center and residential areas. This beach is situated next to the estuary of the Silandak River [1]. The area features many mangrove trees deliberately planted to combat coastal erosion. Additionally, there are many fish farming ponds along the Tirang Coast. Tirang Beach is one area used to set up ponds in coastal regions. Traditional ponds at Tirang Beach are managed using traditional systems, typically characterized by basic technology and reliance on sea tides for water exchange. These ponds are maintained by the local community. Each pond generally has an irregular shape and is stocked with several types of farmed fish, such as milkfish, deep-bodied mojarra fish, saline tilapia, white snapper, spotted catfish, sembilang fish, and mullet fish. Tirang Beach also has mangrove forests and numerous ponds, including shrimp and milkfish ponds [2]. Various human activities at Tirang Beach have led to polluted wastewater. This water may contain many pathogenic bacteria that can cause diseases that affect aquatic organisms if it is not properly managed [3,4]. Waste-containing pathogenic bacteria can be carried into the sea through the drainage systems of Semarang City and the Tapak River. A previous study found a gene related to antibiotic resistance to Imipenem, Tetracycline, and Methicillin from the bacterial communities in the sediment in Tugu, Semarang, where the mangrove ecosystem was converted into aquaculture ponds and continues to re-plant the mangrove since 1990 [5]. In 2001, Tendencia and de la Peña obtained bacteria from ponds with the highest resistance to oxytetracycline at 4.3% of the total number of isolates [6].

One method to combat pathogenic bacteria is the use of antibiotics, which is an increasing trend; however, caution is necessary because inappropriate and excessive doses can lead to resistance. Overuse of antibiotics can cause bacteria to become resistant to them. These bacteria can be found in various locations. Most studies investigate water, sediments, and other abiotic factors; however, there is a lack of studies to compare abiotic and biotic factors [6]. Yuan and colleagues stated that there is no pattern yet in antibiotic-resistant bacteria associated with organisms and the environment as well as the overuse of antibiotics can leave residues and development of antibiotic resistance bacteria (ARB) also antibiotic resistance genes (ARGs) [7]. Hence, this research aimed to determine the level of resistance of these bacteria to several types of antibiotics in the biotic and abiotic, e.g., fish and sediment. This study also identified the most resistant bacteria to bring recent information on the evolution of bacteria becoming resistant.

2. Materials and method

2.1. Study site

The tools used in this research consisted of field sampling tools and sample analysis. The following tools were used in sampling: a GARMIN GPS to determine the sampling point, sediment core that is used to take sediment samples, stationery to record the results obtained in the laboratory or in the field, a camera for documentation at the sampling location, a DO meter AR8210 Smart Sensor to measure temperature, a Morinome pH meter to measure pH and temperature at the sampling locations, an ATC refractometer to measure water salinity, a Secchi disk to measure brightness, a scale stick to measure depth, a plastic zipper to hold samples from the waters, and a cool box to store samples temporarily. The tools used for sample analysis were petri dishes to make media that will be used for isolating the bacteria, measuring cups to measure the volume of a solution, analytical scales to measure the amount of material, autoclaves for sterilizing tools, test tubes for diluting samples, test tube racks, a Bunsen to minimize tool contamination, a vortex for homogenizing samples, a hot plate magnetic stirrer for homogenizing media, a micropipette and microtip for taking material in the form of a solution in small volumes, a tube needle for transferring bacterial isolates to the media, cotton swabs for taking bacterial suspension, a spray bottle for rinsing the slide during Gram staining, a slide for placing bacterial cultures in the Gram staining test, a dropper pipette for transferring the solution, a microscope for observing bacteria, tweezers for taking antibiotic discs, a BIOBASE Biosafety Cabinet (BSC) to carry out sample

planting and pouring media, label paper to mark the sample, plastic wrap to wrap the petri dish to make it tighter, and a caliper to measure the diameter of the clear zone. The test materials used in this research were sediment and fish samples, the other wet laboratory materials consist of Nutrient Broth (NB) and Agar Powder for bacterial culture media, nystatin for anti-fungal on agar media, distilled water for instrument calibration and sample dilution, antibiotic discs for testing antibiotic resistance, a Gram Kit for Gram staining test (HIMEDIA), a PCR kit MyTaq™ Mix Bioline for performing amplifications, and 70% alcohol for sterilizing equipment. The sediment and fish samples were obtained from ponds in the Tirang Beach area. The sampling points were in a traditional mixed fish pond. The samples were taken on January 8th, 2024, using a random sampling method at three points (point 1: 6°57'17.60447"S and 110°21.'16.34285"E; point 2: 6°57'17.49175"S and 110°21'14.08104"E; point 3: 6°57'15.5"S and 110°21'14.1"E). Sediment samples were taken using sediment cores at a depth of 10 cm. The samples were put in plastic zipper bags, placed in a cool box, and taken to the laboratory for analysis. Three fish samples were taken in one collection. The fish chosen were between 7-10 cm long. The samples were then put in plastic zipper bags, placed in a cool box, and taken to the laboratory for analysis. Sample analysis was conducted at the Water Quality Laboratory, Faculty of Fisheries and Marine Sciences, Universitas Diponegoro, Semarang.

2.2. Sterilization of tools and materials

Sterilization is an important stage for killing microorganisms on laboratory equipment to eliminate contaminants. The tools and materials were sterilized via the wet sterilization method with an autoclave at a temperature of 121°C and a pressure of 2 atm for 20 minutes [8]. This method was used to sterilize media, liquids, and laboratory equipment. The items sterilized included petri dishes, test tubes, seawater, microtips, and agar media. Tools made of glass, such as Petri dishes and test tubes, were wrapped in paper before being put into the autoclave.

2.3. Media Culture Preparation

The media used was Nutrient Agar (NA), composed of NB (6.5 gr) and Agar Powder (7.5 gr) dissolved in 500 mL of seawater. The solvent used was seawater, with salinity adjusted to the environmental salinity at each point, which was 25%. The media was sterilized in an autoclave for 20 minutes at 121°C. After autoclaving, the media was supplemented with 75 μg/mL of nystatin as an antifungal agent [6]. The media was then allowed to cool slightly to a temperature of around 40-45°C before being poured into sterile Petri dishes.

2.4. Serial dilution

Serial dilution was carried out to reduce the number of microbes in the liquid, making calculations easier [9]. The sample dilution stage was carried out until it reached the 10^{-5} series. Dilution used sterile seawater with a salinity of 25%. Fish samples were taken from the gills and then crushed using a pestle and mortar. Samples of sediment and fish gills, each weighing 1 gr, were placed in a test tube containing 9 mL of sterile seawater [10]. The second test tube was filled with 9 mL of sterile seawater. This process was repeated until a 10^{-5} dilution series was obtained.

2.5. Bacterial Isolation

The sediment and gill bacteria were isolated using the pour plate method on Nutrient Agar (NA) media. The pour plate method was used to obtain pure cultures with less risk of contamination [11]. The results of the dilution of sediment and gill samples in series 10^{-4} and 10^{-5} were each taken 1 mL and inoculated into a Petri dish aseptically. Then, the NA medium was poured into the Petri dish. The Petri dish was gently swirled in a figure eight motion to mix the bacteria and media homogeneously [10]. The isolation results were placed in an incubator for 24 hours at 37°C [12].

2.6. Total Plate Count (TPC)

Total Plate Count (TPC) is a method for growing live microbial cells in media so that the cells thrive and form macroscopically visible colonies. Colony counts were performed using a hand counter to facilitate the process [10]; 30 and 300 bacterial colonies were counted. Plates containing <300 colonies were not counted for TPC because they

were considered Too Numerous To Count (TNTC). Similarly, samples containing fewer than 30 colonies were not counted because they did not meet the requirements. The results of the bacterial colony counts were then calculated with the formula 1 [13]:

$$N = \text{Colony per petri dish} \times \frac{1}{\text{dilution factor}} \times \text{inoculum volume (mL)} \quad (1)$$

Information:
N = Colony density per sample in Colony Forming Units (CFU/mL)

2.7. Bacterial purification

Purification was performed to separate bacterial colonies with different morphologies into single isolates. The bacteria that grew were differentiated based on variations in colony morphology, including size, shape, color, and elevation [14]. Selected colonies were cultured and purified according to their respective characteristics. The isolates were then selected and streaked onto Nutrient Agar (NA) media using a streak plate technique with a T scratch type using a loop needle. Each plate was used for the growth of one type of bacteria. The bacteria were then incubated for 24 hours at 37°C.

2.8. Antibiotic resistance test

Bacterial isolates were tested with Tetracycline and Chloramphenicol at a concentration of 30 µg, Erythromycin at 15 µg, and Ciprofloxacin at 5 µg. These four antibiotics were chosen because of their wide use in aquaculture and the abundance of their antibiotic resistance gene in most places, including South Korea, China, Sri Lanka, and Italy [7]. The purified bacteria were grown on agar slant media, which was placed in tilted test tubes and inoculated in a zigzag manner. This process aimed to revitalize the bacteria and create a second culture storage stock. Bacterial cultures were then incubated for 24 hours at 37°C [9]. The bacterial culture was taken and placed in 9 mL of sterile seawater with a salinity of 25%. It was then homogenized using a vortex, and the turbidity was visually compared with 0.5 McFarland solution. The purpose of this comparison was to standardize estimates of the number of bacteria to be studied. McFarland 0.5 was equivalent to 1.5 x 10^8 CFU/mL [15]. Incubation was then carried out for 24 hours at 37°C. The inhibition zone formed around the disc was measured in mm for both vertical and horizontal diameters. The diameter of the inhibition zone grouped bacteria into three categories: sensitive, intermediate, and resistant to antibiotics. The inhibition zone formed was classified into three criteria shown in Table 1, and the inhibition zone was calculated by Formula 2.

Table 1. Bacterial inhibition zone criteria.

Antibiotic	Diameter zone		
	R	I	S
Tetracycline [16]	≤14	15-18	≥ 19
Erythromycin [16]	≤13	14-17	≥ 18
Chloramphenicol [16]	≤12	13-17	≥ 18
Ciprofloxacin [17]	≤ 15	16-19	≥ 20

The following formula was used for calculating the inhibition zone [18]:

$$\text{Inhibition zone} = \frac{\{(DV-DC)+(DH-DC)\}}{2} \quad (2)$$

where DV is the vertical diameter, DH is the horizontal diameter, and DC is the disc diameter.

2.9. Gram stain test

The Gram staining test was carried out on bacteria that form a clear zone on the agar medium. This test was done according to the Gram Stain-Kit instructions by taking bacterial isolates with a circular needle and streaking them on a glass object. Then, crystal violet was added for one minute, followed by washing with sterile distilled water and drying. Furthermore, the bacterial isolate was dropped onto Lugol and left for one minute before washing again with sterile distilled water. The bacterial isolate was then subjected to the Gram Decolorizer for five seconds until the crystal violet no longer dissolved, followed by washing again with sterile distilled water. Finally, safranin was added to the bacterial isolate and left for 45 seconds before being washed with sterile distilled water, dried, and examined under a light microscope with 1000 times magnification.

2.10. Molecular identification

Molecular identification was carried out through DNA extraction, DNA amplification, and DNA visualization. DNA extraction was done by placing bacterial isolates in a microtube containing 250 µL of 10% Chelex. The microtube was then

homogenized with a vortex for 20 seconds. Moreover, it was centrifuged at 13,000 rpm for 15 seconds. The microtubes were then placed on a heating block at 95°C for 45 minutes. After heating, the sample was vortexed again for 20 seconds and centrifuged at 13,000 rpm for 15 seconds. The supernatant was extracted with a micropipette and transferred to a new microtube. Amplification was performed using the Polymerase Chain Reaction (PCR) technique. Initially, 1 µL of template DNA or extracted DNA was mixed with the PCR mix in a PCR tube. The PCR mix consisted of 12.5 µL of Mytaq DNA, 1 µL each of forward primer 27F and reverse primer 149R, and 9.5 µL of ddH2O. The PCR tube was then gently shaken and placed in the thermal cycler. Amplification conditions were set as follows: pre-denaturation at 95°C for three minutes, denaturation at 95°C for one minute, annealing at 53.9°C for one minute, extension at 72°C for one minute, and final extension at 72°C for seven minutes, for a total of 30 cycles [19]. Electrophoresis was carried out by preparing a 1% agarose gel, which was made by dissolving 0.4 g of agarose in 40 mL of Tris-Boric EDTA (TBE) and heating it on a hotplate until it boiled. After adding 3 µL of a green gel dye, the agarose was poured into a gel mold and allowed to harden. The 1% agarose gel was then placed in an electrophoresis tank and submerged in 1X TBE. Electrophoresis was performed for 30 minutes at 100 volts and 400 milliamps. The first well was filled with a mixture of 2 µL of Ladder DNA and 1 µL of Loading Dye as a marker, while the subsequent wells were filled with a mixture of 2 µL DNA template and 1 µL Loading Dye. The agarose gel from electrophoresis was then inserted into a UV Doc for DNA visualization. The DNA visualization results were sent to PT. Genetic Science, Jakarta, to determine the 16S RNA gene sequence. The results were aligned with nucleotide sequences using the MEGA 11.0 application. Sequencing was used to identify genes by comparing them with sequence data in GenBank [20]. The GenBank used is the National Center for Biotechnology Information (NCBI). Nucleotide sequences showing similarity were further analyzed using the Basic Local Alignment Search Tool (BLAST) to compare the target sequence with existing database sequences and identify similar ones.

3. Result and discussion

3.1. Abundance of bacteria

Deep-bodied mojarra fish (*Gerres erythrourus*) were used in this study. According to the obtained results, the highest TPC value from the sediment samples came from Point 3, with an average of 5.52 x 10^6 CFU/mL; the lowest value was at Point 2, which was 7.2 x 10^5 CFU/mL (Table 2). Sample I.B.2 was deemed ineligible for counting using TPC due to having a total colony of <30 colonies. Meanwhile, sample I.A.1 was classified as TNTC because the value was >300 colonies. The TPC values for sediment samples indicated that the highest TPC value was observed from Point 2, namely 2.45 x 10^7 CFU/mL, while the lowest value was at Point 3, with abundance at 1.53 x 10^6 CFU/mL (Table 3). Sample S.A.2 was classified as TNTC. From the result, the sediment bacteria have more abundance rather than in fish gills. The similar findings also found from Yuan and colleagues' studies [7].

Table 2. Calculation results of Total Plate Count on fish samples.

Point	Sample code	Dilution	Number of Colonies	Total Plate Count (CFU/mL)
1	I.A.1	10^{-4}	371	TNTC
	I.B.1	10^{-5}	34	3.40 x 10^6
Average TPC (CFU/ mL)				3.40 x 10^6
2	I.A.2	10^{-4}	72	7.20 x 10^5
	I.B.2	10^{-5}	29	-
Average TPC (CFU/ mL)				7.20 x 10^5
3	I.A.3	10^{-4}	104	1.04 x 10^6
	I.B.3	10^{-5}	100	1.00 x 10^7
Average TPC (CFU/ mL)				5.52 x10^6

I: fish gills; A:10^{-4} dilution; B: 10^{-5} dilution; numbers (1,2,3): sampling point; TNTC: Too Numerous to Count

Table 3. Calculation results of Total Plate Count on sediment samples.

Point	Sample code	Dilution	Number of Colonies	Total Plate Count (CFU/mL)
1	S.A.1	10^{-4}	170	1.70×10^{6}
	S.B.1	10^{-5}	243	2.43×10^{7}
Average TPC (CFU/ mL)				1.30×10^{7}
2	S.A.2	10^{-4}	353	TNTC
	S.B.2	10^{-5}	245	2.45×10^{7}
Average TPC (CFU/ mL)				2.45×10^{7}
3	S.A.3	10^{-4}	240	2.40×10^{6}
	S.B.3	10^{-5}	66	6.60×10^{6}
Average TPC (CFU/ mL)				1.53×10^{6}

S: sediment; A:10^{-4} dilution; B: 10^{-5} dilution; numbers (1,2,3): sampling point; TNTC: Too Numerous to Count

3.2. Antibiotic resistance test

The activity of the inhibition zone formed from the resistance test against the four antibiotics got different results for each bacterial isolate. The most resistant bacterial isolate in the sediment was tested with Tetracycline, as evidenced by the inhibition zone of 7.3 mm in isolate S.A.2.b (Table 4). The most resistant fish isolate was tested with Erythromycin with an inhibition zone of 0 mm on isolate I.A.1.d. Differences in the diameter of the inhibition zone can be caused by the ability of antibiotics to inhibit or kill bacterial growth will be different between each isolate. Varied diameters of inhibition zones indicate distinct levels of resistance to antibiotics for each bacterium. A smaller zone of inhibition suggests a higher level of resistance to antibiotics in the bacterial isolate. Conversely, bacterial isolates that produce larger inhibition zone diameters are unable to impede the performance of antibiotics in killing or inhibiting their growth.

The inhibition zone formed signifies the antibiotic's capability to inhibit microbial protein synthesis and its bacteriostatic nature [21]. Bacterial resistance can occur due to two factors: primary factors and supporting factors. Primary factors are the use of antibiotic agents, the emergence of antibiotic-resistant bacterial strains, and the dissemination of resistant bacterial strains to other bacteria. Supporting factors encompass the location of infection, the effectiveness of antibiotics in reaching the target organ of infection at therapeutic concentrations, and environmental ecology [22].

The results showed that the most resistant bacterial isolates were tested with Ciprofloxacin, while the most intermediate was found with Tetracycline (Figure 1; Table 4). The most sensitive isolates were observed with Chloramphenicol. In three out of the four antibiotics tested, fish isolates exhibited a higher number of resistant isolates. Isolates tested with Chloramphenicol showed an equal number of resistant isolates in both types of samples (Figure 2). The most resistant fish isolates were observed in the tests with Ciprofloxacin. Isolates that demonstrated the highest resistance to three out of the four types of antibiotics tested were predominantly from fish gill samples. This phenomenon is believed to be due to the long-term use of antibiotics, enabling bacteria in the fish's body to adapt and develop resistance. Resistance occurs when bacteria undergo gene mutations or acquire mutated genes from other organisms that encode resistance in response to antibiotic use [23]. Furthermore, gills are among the organs where bacteria are most readily found. Gills serve as the primary target for infectious diseases and antibiotic treatment due to their function as water filters for oxygen acquisition [24]. Unlike fins and tails, which serve as means of locomotion, bacteria can easily attach to gills [25]. Gills have lamellae that play a crucial role in oxygen filtration while also serving as a pathway for pathogens to enter and infect them, as organic material present in the gills can serve as a food source for these pathogens [26]. Gill infections are more prevalent than in other organs due to the exposure of these organs to microbiota [27]. The highest number of resistant bacterial isolates were observed in tests with Ciprofloxacin. The presence of resistant isolates is believed to be due to the prolonged use of Ciprofloxacin, enabling bacteria in the body to adapt and develop resistance to antibiotics. Ciprofloxacin functions by inhibiting two bacterial

enzymes: DNA gyrase and topoisomerase IV [28]. Additionally, the antibiotic Ciprofloxacin is a type of antibacterial that is frequently used to treat diseases due to its broader spectrum of action in treating bacterial infections [29]. The increased use of antibiotics raises the likelihood of pathogenic bacteria modifying their cell structure to counteract antibiotic interactions, leading to antibiotic resistance [30]. Conversely, the highest number of bacterial isolates in the sensitive category were observed in tests with the antibiotic Chloramphenicol. This high level of sensitivity may be attributed to the infrequent use of this antibiotic in treatment, as indicated by research by Sasongko [31]. They found that Chloramphenicol had the lowest level of resistance to *E. coli* compared to other antibiotics tested.

Table 4. Results of inhibitory zone measurements in sediment and fish gill bacterial isolates.

Isolate	Inhibition Zone							
	TE 30 µg (mm)	Category (*)	E 15 µg (mm)	Category (*)	C 30 µg (mm)	Category (*)	CIP 5 µg (mm)	Category (*)
S.A.1.a	22.68	S	22.83	S	28.08	S	21.07	S
S.A.2.a	14.48	I	23.87	S	16.67	I	11.74	R
S.A.2.b	7.30	R	1.74	R	19.73	S	9.55	R
S.A.3.a	15.52	I	24.90	S	8.30	R	21.35	S
S.B.1.a	25.33	S	27.62	S	26.97	S	16.97	I
S.B.1.b	19.92	S	25.61	S	22.50	S	13.10	R
S.B.1.c	23.77	S	27.32	S	30.39	S	17.05	I
S.B.1.d	21.46	S	26.58	S	24.48	S	16.12	I
S.B.2.a	23.51	S	26.48	S	21.08	S	14.54	R
S.B.3.a	28.97	S	32.67	S	19.49	S	18.88	I
S.B.3.b	18.24	I	21.75	S	26.12	S	18.98	I
S.B.3.c	23.53	S	27.05	S	23.60	S	16.57	I
S.B.3.d	26.81	S	28.34	S	33.24	S	20.89	S
S.B.3.e	25.00	S	25.07	S	26.45	S	22.02	S
I.A.1.a	19.68	I	33.77	S	34.44	S	10.83	R
I.A.1.b	12.20	R	21.95	S	21.56	S	11.83	R
I.A.1.c	14.14	I	3.34	R	19.65	S	12.18	R
I.A.1.d	16.39	I	0.00	R	22.20	S	9.27	R
I.A.2.a	13.01	R	3.81	R	19.72	S	7.56	R
I.A.3.a	10.28	R	15.09	I	24.64	S	22.84	S
I.B.1.a	12.88	R	16.70	I	31.52	S	23.02	S
I.B.1.b	10.89	R	5.74	R	20.70	S	16.64	I
I.B.1.c	12.42	R	3.98	R	18.83	S	13.5	R
I.B.2.a	10.33	R	3.60	R	19.41	S	8.17	R
I.B.2.b	14.16	I	22.98	S	22.86	S	11.66	R
I.B.2.c	12.29	R	23.18	S	9.64	R	11.23	R
I.B.3.a	15.05	I	2.46	R	19.99	S	8.03	R
I.B.3.b	12.63	R	22.92	S	23.07	S	9.90	R
I.B.3.c	13.67	R	2.88	R	19.50	S	8.48	R
I.B.3.d	14.42	I	25.07	S	22.94	S	10.92	R

S: Sediment; I: fish gills; A:10^{-4} dilution; B:10^{-5} dilution; numbers (1,2,3): sampling point; (a,b,c,d): pure isolate; R: Resistant; I: Intermediet; S; Sensitive: TE: Tetracycline; C: Chloramphenicol; CIP: Ciprofloxacin; E: Erythromycin; (*)= CLSI, 2021.

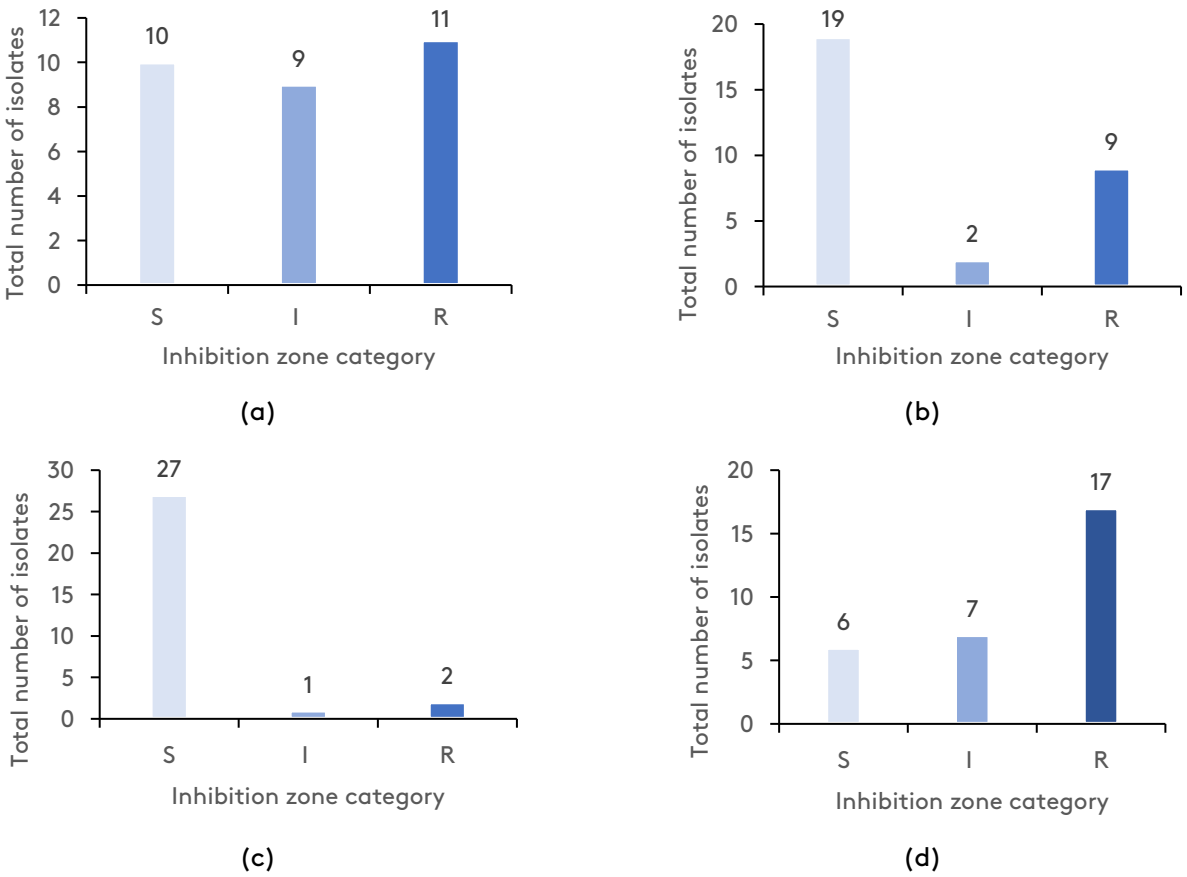

Fig. 1. Categories of inhibition zones formed in bacteria against (a)Tetracycline, (b) Erythromycin, (c) Chloramphenicol, and (d) Ciprofloxacin.

3.3. Gram staining

Based on the results of the Gram staining test on the most resistant isolates, both types of isolates exhibited red bacilli, indicating that the bacteria were Gram-negative (Figure 3). Bacilli-shaped bacteria are the most abundant bacteria in nature [32]. This finding aligns with research by Kamelia et al. [26], which stated that 64.7% of the tested bacteria exhibited bacilli morphology, while the remaining were cocci. Gram-negative bacteria are unable to retain the iodine color when exposed to alcohol. They possess cell walls containing small amounts of peptidoglycan compared to Gram-positive bacteria, but they have an outer membrane comprising lipoproteins, phospholipids, and lipopolysaccharides, rendering them less resistant [33]. The lipopolysaccharide content also makes Gram-negative bacteria more virulent than Gram-positive bacteria. Gram-negative bacteria can thrive in environments containing nitrogen and phosphorus originating from farmed fish waste and leftover feed. Generally, Gram-negative bacteria are pathogenic in fish [34].

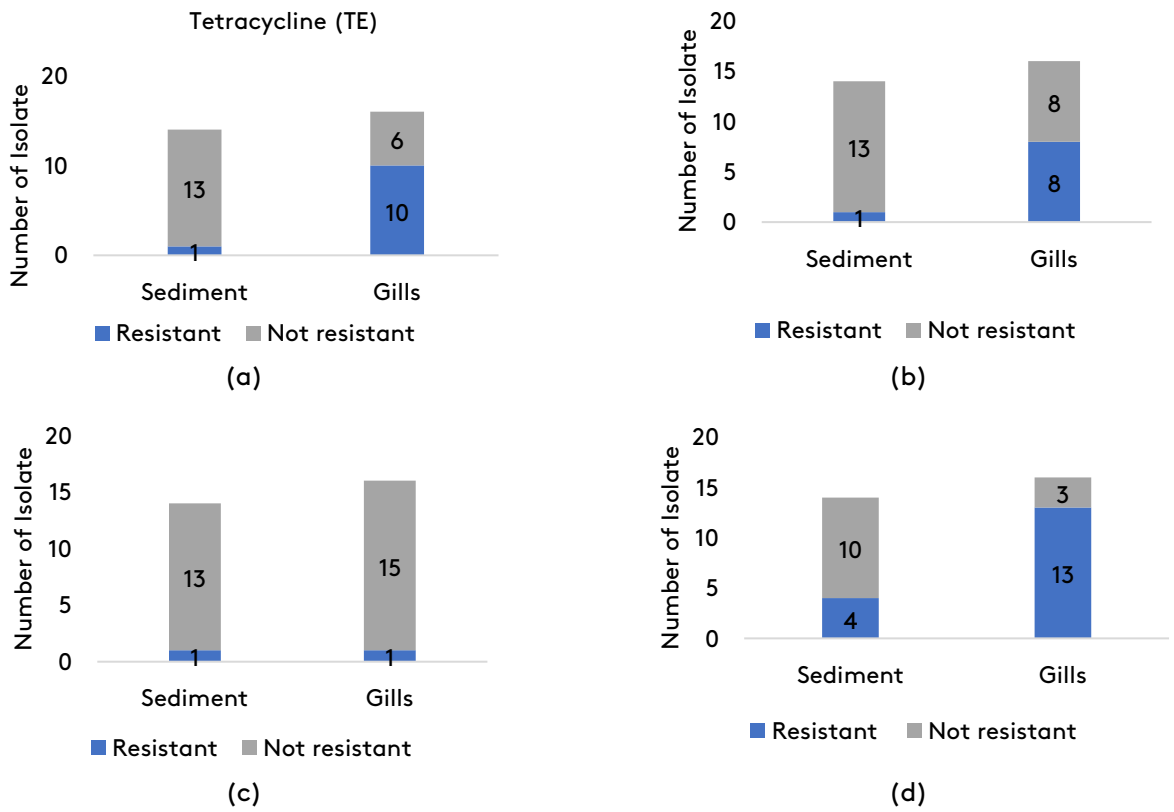

Fig. 2. Comparison of resistant and non-resistant isolates (a) Tetracycline, (b) Erythromycin, (c) Chloramphenicol, and (d) Ciprofloxacin.

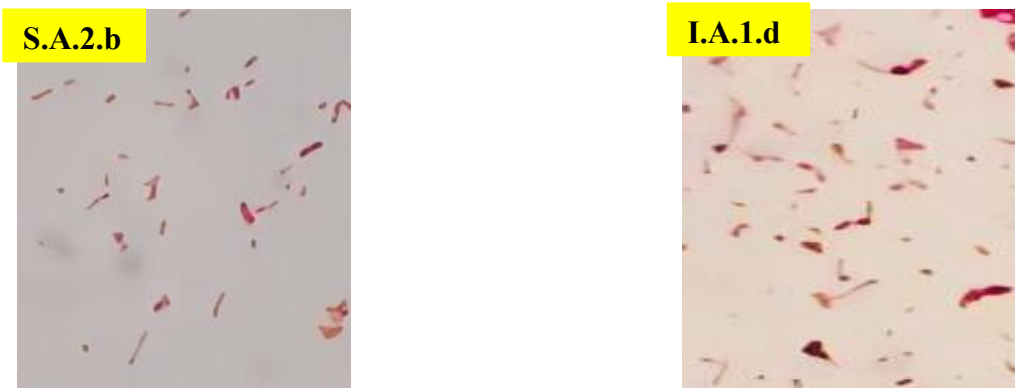

Fig. 3. Gram staining results of the most resistant bacteria in 1,000 X magnification (S.A.2.b: sediment bacterium; I.A.1.d: fish bacterium).

3.4. Resistant isolate

The most resistant isolates in fish and sediment were subjected to PCR to determine the species. The electrophoresis results indicated that bacterial DNA fragments from both sediment and fish samples had a size of 1,500 base pairs (Figure 4). The size of an organism's genomic DNA varies depending on the organism. Prokaryotic organisms generally have smaller genomes, both in terms of base pairs and the number of genes, compared to eukaryotic organisms [35]. The sequencing results of each forward and reverse primer were then combined by reversing the sequencing results to perform pairwise reversal (reverse complement). The base sequences obtained from the sequencing results were then combined and analyzed using the BLAST program. The results of 16S rRNA sequence analysis using the BLAST program indicated that the most resistant fish isolate exhibited 100% similarity to *Vibrio alginolyticus,* while the most

resistant sediment isolate exhibited 99.20% similarity to *Vibrio parahaemolyticus* (Table 5).

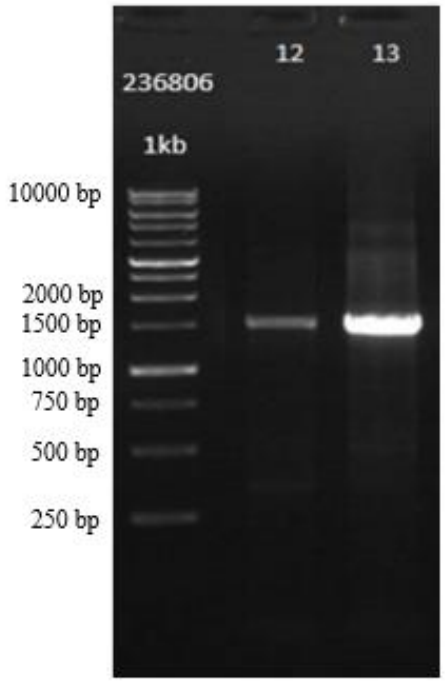

Fig. 4. Results of DNA electrophoresis of fish and sediment bacterial genomes; 12: sample S.A.2.b; 13: sample I.A.1.d.

Based on the phylogenetic tree, which includes several sequence data from the NCBI GenBank for comparison, it is evident that the bacteria from the most resistant fish isolate (I.A.1.d) were on the same branch as *Vibrio alginolyticus* (Figure 5). This bacterium is Gram-negative and has a rod shape. It is commonly found in marine environments due to its ability to survive high salt levels. *V. alginolyticus* is a pathogenic bacterium that contaminates aquaculture biota [36]. It often attacks fish when their immune system is weak, leading to infections and injuries that may result in death [37]. Moreover, this bacterium can cause eye, ear, diarrhea, and opportunistic infections in humans [38]. Similarly, the most resistant sediment isolate (S.A.2.b) was on the same branch as *Vibrio parahaemolyticus* with a bootstrap value of 99% (Figure 6). The numbers on each branch of the tree show the bootstrap value [35]. *Vibrio parahaemolyticus* is an aquatic bacterium belonging to the Vibrionaceae family. *V. parahaemolyticus* is Gram-negative and has a rod shape. This bacterium is categorized as quite dangerous pathogenic bacteria. *V. parahaemolyticus* primarily attacks farmed fish, such as shrimp, and is one of the leading causes of disease in shrimp, notably as the causative agent of Acute Hepatopancreatic Necrosis Disease [39]. *V. parahaemolyticus* can also be pathogenic in the human body because it contains the toxin Thermostable Direct Hemolysin (TDH) or Thermostable Direct Hemolysin Related Hemolysin (TRH), which can cause nausea, vomiting, stomach cramps, fever, and watery to bloody diarrhea. *V. parahaemolyticus* can contaminate cultivated biota; if consumed by humans, it can cause disease or poisoning [40]. Quantitatively, more than one-third of the bacterial isolates tested exhibited resistance to Tetracycline and Ciprofloxacin. This finding indicates the residuals antibiotic in the environment could trigger resistance in bacteria, even though in the traditional pond is absence of antibiotics usage. Regular monitoring of water quality and disease surveillance can help minimize bacterial infections. Government intervention in controlling and monitoring the use and sale of antibiotics is also essential. Additionally, training sessions for drug sellers, feed distributors, and farmers on increasing awareness of the dangers of antibiotics will contribute to reducing their use in aquaculture. Monitoring antibiotics in aquaculture will facilitate a better understanding of their usage and associated factors [41]. Rational use of antibiotics is necessary to prevent the spread of antibiotic-resistant bacteria [42].

Table 5. BLAST results of fish and sediment DNA isolates.

Sample Code	Species	Score	Query Coverage	Per. Identity	Acc. Number
Fish Gills (I.A.1d)	*Vibrio alginolyticus* strain FBC23-131	750	100%	100.00%	OR875846.1
Sediment (S.A.2.b)	*Vibrio parahaemolyticus* strain CZB-31	449	99%	99.20%	KT023512.1

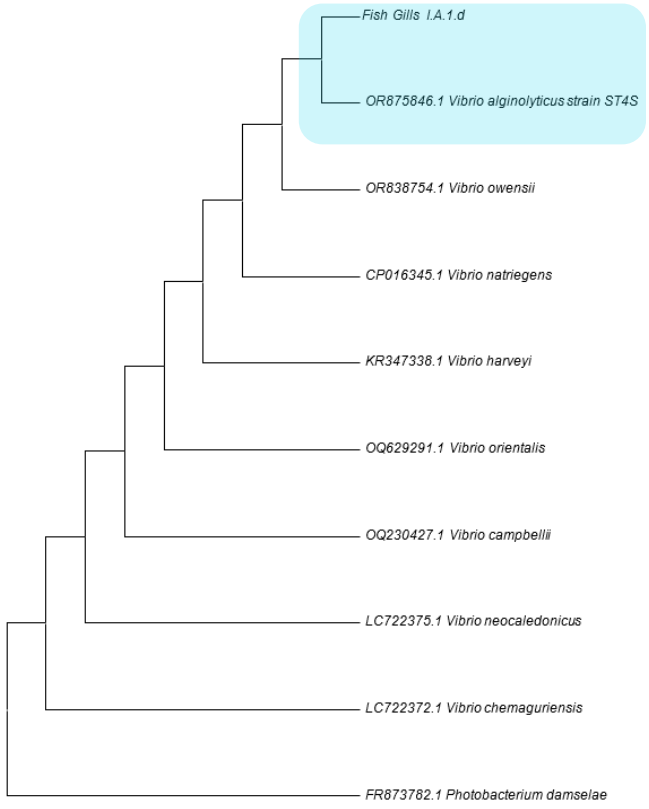

Fig. 5. Tree phylogenetic of antibiotic-resistant fish bacterial 16S rRNA genes (Neighbor-Joining construction using 1000 bootstrap).

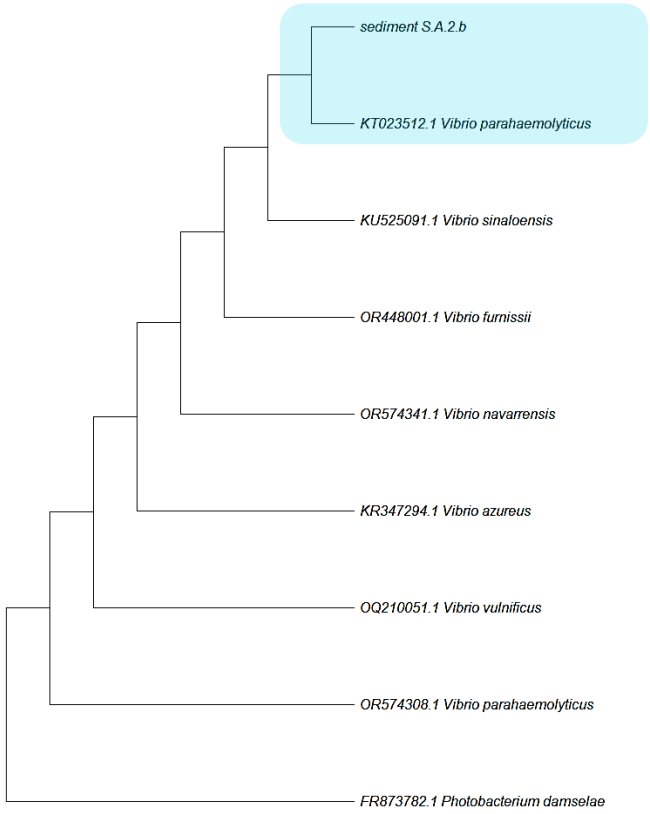

Fig. 6. Tree Phylogenetic of antibiotic-resistant sediment bacterial 16S rRNA genes (Neighbor-Joining construction using 1000 bootstrap).

4. Conclusion

Based on the results, the abundance of bacteria in the sediment and fish gills from the traditional ponds in the Tirang Beach area was 1.53 x 10^6 to 2.45 x 10^7 CFU/mL and 7.20 x 10^5 to 5.52 x 10^6 CFU/mL, respectively. The morphological characteristics of sediment and fish gill bacterial isolates were macroscopically dominated by a circular shape, with the dominant color being milky white, elevation being raised, and entire edges. Overall, the highest resistance for all the isolates comes from Ciprofloxacin. The most resistant bacteria in fish gill identified as *Vibrio alginolyticus* type, a rod-shaped Gram-negative bacterium, while the most resistant sediment bacteria found were recognized as *Vibrio parahaemolyticus* from the 16S rRNA gene.

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References

[1] Prahmani, Y.S., Deanova, D., Fariz, T.R., & Heriyanti, A.P. (2022). The Impact of Abrasion in the Tirang Coastal Area on the Physical Environment in Tugu District. Journal of Urban and Regional Planning, 3(2), 52-59. http://dx.doi.org/10.26418/uniplan.v3i2.56675

[2] Sinulingga, H.A., Muskananfola, M.R., & Rudiyanti, S. (2018). The Relationship between Sediment Texture and Organic Matter with Macrozoobentos in Mangrove Habitat of Tirang Beach, Semarang. Management of Aquatic Resources Journal, 6(3), 247-254. https://doi.org/10.14710/marj.v6i3.20583

[3] Pandey, P. K., Kass, P. H., Soupir, M. L., Biswas, S., & Singh, V. P. (2014). Contamination of water resources by pathogenic bacteria. AMB Express, 4, 51. https://doi.org/10.1186/s13568-014-0051-x

[4] Su, Y., Gao, R., Huang, F., Liang, B., Guo, J., Fan, L., Wang, A., & Gao, S.H. (2024). Occurence, transmission and risks assessment of pathogens in aquatic environments accessible to humans. Journal of Environmental Management, 354:120331. https://doi.org/10.1016/j.jenvman.2024.120331

[5] Sabdaningsih, A., Adyasari, D., Suryanti, S., Febrianto, S., & Eshananda, Y. (2023). Environmental legacy of aquaculture and industrial activities in mangrove ecosystems. Journal of Sea Research, 196:102454. https://doi.org/10.1016/j.seares.2023.102454

[6] Tendencia, E.A., & de la Peña, L.D. (2001). Antibiotic resistance of bacteria from shrimp ponds. Aquaculture, 195(3-4):193-204. https://doi.org/10.1016/S0044-8486(00)00570-6

[7] Yuan, X., Lv, Z., Zhang, Z., Han, Y., Liu, Z., & Zhang, H. (2023). A Review of Antibiotics, Antibiotic Resistant Bacteria, and Resistance Genes in Aquaculture: Occurrence, Contamination, and Transmission. Toxics, 11, 420. https://doi.org/10.3390/toxics11050420

[8] Wulandari, S., Nisa, Y. S., Taryono, T., Indarti, S. dan Sayekti, R. S. (2021). Sterilization of Tissue Culture Equipment and Media. Agrotechnology Innovation, 4(2):16-19. https://doi.org/10.22146/a.77010

[9] Rosmania, R., & Yanti, F. (2020). Calculation of the Number of Bacteria in Microbiology Laboratory Using the Development of Spectrophotometry Method. Jurnal Penelitian Sains, 22(2):76-86. https://doi.org/10.56064/jps.v22i2.564

[10] Tyas, D. E., WIdyorini, N., & Solichin, A. (2018). Differences in the Amount of Bacteria in Sediment in Mangrove and Non-Mangrove Areas in the Waters of Bedono Village, Demak. Management of Aquatic Resources Journal, 7(2):189-196. https://doi.org/10.14710/marj.v7i2.22541

[11] Kurniawan, S.Y., & Ariami, P. (2023). Si Pinter as a Counting Tool for Bacterial Colonies Supporting Microbiology Laboratories. Jurnal Biotek, 11(1):88-98. https://doi.org/10.24252/jb.v11i1.35436

[12] Nazhifah, A. K., Furtuna, D.K., Praja, R. K., Martani, N. S., & Ratnasari, A. (2024). Analysis of Total Plate Counts of Bacteria and the Presence of *Salmonella typhi* in Chili Sauce of Traders' Snacks in the University of Palangka Raya. Tropis: Jurnal Riset Teknologi Laboratorium Medis, 1(1):25-44. https://e-journal.upr.ac.id/index.php/tropis/article/view/11885

[13] Utami, S., Bintari, S. H., & Susanti, R. (2018). Detection of *Escherichia coli* in Jamu Gendong in Gunungpati With Differential Selective Medium.. Life science, 7(2):73-81. https://journal.unnes.ac.id/sju/UnnesJLifeSci/article/view/26968

[14] Wantania, L. L., Ginting, E. L., & Wullur, S. (2016). Isolasi Bakteri Simbion dengan Spons dari Perairan Tongkeina, Sulawesi Utara. Jurnal LPPM Bidang Sains dan Teknologi, 3(1):57-65. https://doi.org/10.35801/jlppmsains.3.1.2016.15208

[15] Aviany, H.B., & Pujiyanto, S. (2020). Analysis of the Effectiveness of Probiotics in Beauty Products as Antibacterial against *Staphylococcus epidermidis* Bacteria. Berkala Bioteknologi, 3(2):24-30. https://ejournal2.undip.ac.id/index.php/bb/article/view/9657

[16] Soleha, T. U. (2015). Antibiotic Sensitivity Test. Juke Unila, 5(9):119-123. http://download.garuda.kemdikbud.go.id/article.php?article=328320&val=5503&title=Uji%20Kepekaan%20terhadap%20Antibiotik

[17] Muslim, Z., Novrianti, A., & Irnameria, D. (2020). Resistance test of bacterial causes of urinary tract infection against ciprofloxacin and ceftriaxone antibiotics. *SANITAS:* Jurnal Teknologi Dan Seni Kesehatan, 11(2), 203-212. https://doi.org/10.36525/sanitas.2020.19

[18] Toy, T. S., Lampus, B. S., & Hutagalung, B. S. (2015). Inhibition Test of *Gracilaria* sp. Seaweed Extract Against the Growth of *Staphylococcus*

aureus Bacteria. E-GIGI, 3(1): 153-159. https://doi.org/10.35790/eg.3.1.2015.6600

[19] Ayuningrum, D., Kristiana, R., Asagabaldan, M. A., Sabdono, A., Radjasa, O. K., Nuryadi, H., & Trianto, A. (2017). Isolation, Characterisation and Antagonistic Activity of Bacteria Symbionts Hardcoral *Pavona* sp. Isolated from Panjang Island, Jepara Against Infectious Multi-Drug Resistant (MDR) Bacteria. In IOP Conference Series: Earth and Environmental Science, 55(1):1-8. https://doi:10.1088/1755-1315/55/1/012029

[20] Aris, M., Sukenda, H. E., Fatuhcri, M. S., & Munti, Y. (2013). Molecular identification of pathogenic bacteria and PCR primary design. Jurnal Budidaya Perairan, 1(4): 43-50 https://doi.org/10.35800/bdp.1.3.2013.2733

[21] Dewi, D. G. D. P., Mastra, N., & Jirna, I. N. (2018). Differences in *Staphylococcus aureus* Growth Inhibition Zones in Various Concentrations of Ethanol Extract of Biduri Leaves in Vitro. Meditory, 6(5), 39-45. https://doi.org/10.33992/m.v6i1.227

[22] Pratiwi, R. H. (2017). Mechanisms of Defense of Pathogenic Bacteria against Antibiotics. Jurnal pro-life, 4(3): 418-429. https://doi.org/10.33541/jpvol6Iss2pp102

[23] Anasa, R. B. (2015). Effect of Irrational Antibiotic Use on Bacterial Resistance Rate in Intensive Care Units. Jurnal Agromedicine, 2(4): 486-492. https://juke.kedokteran.unila.ac.id/index.php/agro/article/view/1243

[24] Sukarni, S., Maftuch, M., & Nursyam, H. (2012). Study on the Use of Ciprofloxacin on the Histology of Gill and Liver of Botia Fish (*Botia macracanthus*, bleeker) Infected with *Aeromonas hydrophila* Bacteria. The Journal of Experimental Life Science, 2(1): 6-12. https://doi.org/10.21776/ub.jels.2012.002.01.02

[25] Pusparani, R., Widyorini, N., & Jati, O. E. (2021). Total Analysis of *Aeromonas* sp. on Tilapia (*Oreochromis niloticus*) in the Floating Net Cage (KJA) and Non-KJA Rawa Pening Area. Jurnal Pasir Laut, 5(1): 9-16. https://doi.org/10.14710/jpl.2021.31885

[26] Kamelia, M., Widiani, N., & Adistyaningrum, N. (2018). Analysis of Differences in the Number of Bacteria in Tilapia (*Oreochromis niloticus*) Aquaculture. Biospecies, 11(2): 76-82. https://doi.org/10.22437/biospecies.v11i2.5718

[27] Mzula, A., Wambura, P. N., Mdegela, R. H., & G.M. Shirima. (2019). Phenotypic and Molecular Detection of Aeromonas Infection in Farmed Nile Tilapia in Southern Highland and Northern Tanzania. Heliyon, (5): 1 – 8. https://doi.org/10.1016/j.heliyon.2019. e02220

[28] Tjay, T. H., & Raharja, K. (2015). Essential Medicines, 7th Edition. PT. Elex Media Komputindo, Jakarta http://perpus.fik-unik.ac.id//index.php?p=show_detail&id=5847

[29] Faisal, H., Tarigan, R. E., & Lase, J. (2022). Antibacterial activity of Ag-hydroxyapatite composite of bone-in tuna (*Thunnus albacores*) against *Streptococcus mutans*. Jurnal Sains Natural, 12(2), 78-84. https://doi.org/10.31938/jsn.v%2012i2.378

[30] Hutajulu, I. R., Antara, N. S., & Suhendra, L. (2022). Resistance of Lactic Acid Bacterial Isolate from Asinan Bamboo Bamboo Shoots (*Gigantochloa nigrociliata* Buse – Kurz) to Antibiotics. Jurnal Rekayasa Dan Manajemen Agroindustri, 10(1): 114-123. https://doi.org/10.24843/JRMA.2022.v10.i01.p11

[31] Sasongko H. (2014). Resistance Test of Escherichia Coli Bacteria from Sungai Boyong, Sleman Regency to Antibiotics Amoxicillin, Chloramphenicol, Sulfametoxasol, and Streptomycin. Jurnal Bioedukatika, 2(1): 25-29. https://core.ac.uk/download/pdf/324200569.pdf

[32] Dwidjoseputro. 2012. Fundamentals of Microbiology. UB Press, Jakarta. Dasar-dasar mikrobiologi /D. Dwidjoseputro | OPAC Perpustakaan Nasional RI.

[33] Amin, S.S. Ghozali, T. Z., & Efendi, M. R. S. 2023. Identification of Bacteria from the Palm by Gram Staining. Jurnal Kimia dan Ilmu Lingkungan, 1(1): 30-35. https://doi.org/10.56071/chemviro.v1i1.563

[34] Pękala-Safińska, A. 2018. Contemporary Threats of Bacterial Infections in Freshwater Fish. Journal of veterinary research, 62(3): 261-267. https://doi.org/10.2478/jvetres-2018-0037

[35] Nursyam, H., & Prihanto, A. A. (2018). Molecular Identification of Mangrove Endophytic Bacteria *Rhizophora mucronata* Gelatinase Producing (MMP2). Jurnal Pengolahan Hasil Perikanan Indonesia, 21(1), 143-147. https://doi.org/10.17844/jphpi.v21i1.21537

[36] Liu, X. F., Zhang, H., Liu, X., Gong, Y., Chen, Y., Cao, Y., & Hu, C. (2014). Pathogenic Analysis of *Vibrio alginolyticus* Infection in a Mouse Model. Folia microbiologica, 59, 167-171. https://doi.org/10.1007/s12223-013-0279-x

[37] Tukan, O. B., Salosso, Y., & Djonu, A. (2024). Prevention of *Vibrio alginolyticus* Bacterial Infection in Cantang Grouper Fish (*Epinephelus* sp.) Using Kersen Leaf Decoction (*Muntingia calabura*). Fisheries Journal, 13(3), 634–646. https://doi.org/10.29303/jp.v13i3.55

[38] Zhou, K., Tian, K. Y., Liu, X. Q., Liu, W., Zhang, X. Y., Liu, J. Y., & Sun, F. (2021). Characteristic and Otopathogenic Analysis of a *Vibrio alginolyticus* Strain Responsible for Chronic Otitis Externa in China. Frontiers in Microbiology, 12(750642):1-15 https://doi.org/10.3389/fmicb.2021.750642

[39] TriWanda, A., Hastuti, Y. P., & Rahardjo, S. (2023). Antibacterial Activities of Mangrove Plant Extract (*Rhizophora stylosa*) to the Growth of Bacteria *Vibrio parahaemolyticus*. Aurelia Journal, 5(1), 47-54. http://dx.doi.org/10.15578/aj.v5i1.11650

[40] Vergara., & Jimenez. (2017). Bioactive Components in Moringa Oliefera Leaves Protect Againts Chronic Disease. Antioxidants. 6(4), 91-95. https://doi.org/10.3390/antiox6040091

[41] Chowdhury, N. N., Hicks, E., & Wiesner, M. R. (2022). Investigating and Modeling The Regulation of Extracellular Antibiotic Resistance Gene Bioavailability by Naturally Occurring Nanoparticles. Environmental Science and Technology, 56(21):1-20, https://doi.org/10.1021/acs.est.2c02878

[42] Saudi, A.D.A., & Rusdy. (2018). Antibiotic Resistance Test against Bacteria Causing Urinary Tract Infections at Salewangang Maros Hospital. Media Farmasi, 14(2):27-31. https://doi.org/10.32382/mf.v14i2.587

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