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Detection of Resistance Gene from Aeromonas hydrophila Isolated from Catfish Farming in Jambi

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Abstract: Aeromonas hydrophila is one of the causative agents for Motile Aeromonas Septicemia (MAS) in catfish. MAS treatment is generally carried out by administering antibiotics. This study aimed to determine the antibiotic resistance profile of A. hydrophila and detect the presence of resistance genes. The samples are archived A. hydrophila isolates isolated from catfish ponds in the district of Muara Jambi, Bungo, Jambi Province, and Merangin total of 18 isolates. Testing the antibiotic resistance profile was carried out using the disk diffusion method against tetracycline, oxytetracycline, ampicillin, amoxicillin, ciprofloxacin, enrofloxacin, and streptomycin. The detected resistance genes were ampC, qnrA, qnrB, and qnrS. The results showed A. hydrophila isolates were resistant to tetracycline (100%), oxytetracycline (94.42%), ampicillin (77.70%), and amoxicillin (77.72%) and still sensitive to ciprofloxacin (83.32%), enrofloxacin (100 %), and streptomycin (100%). The ampC gene was found in 8 out of 14 isolates resistant to beta-lactam antibiotics (57.14%). The qnrA and qnrS genes were found in 3 out of 4 isolates resistant to quinolone antibiotics (75%), while qnrB was found in 1 isolate (25%). A. hydrophila isolated from catfish ponds in Jambi develops resistance to antibiotics and resistance genes found in isolates resistant to antibiotics.

Keywords: Aeromonas hydrophila; Antibiotic; Catfish; Gene; Resistance

Introduction

Catfish is a cultivated commodity that has fast growth and has a high ability to adapt to the environment (Hariati et al., 2017). Consumer demand for catfish has increased every year, and this causes catfish production to be increased (Etviliani et al., 2021). Based on statistical data from the Ministry of Marine Affairs and Fisheries, catfish production had decreased from 981,623.40 tons in 2019 to 347,511.48 in 2020. Meanwhile, consumer demand increased in 2018 by 50.69 tons to 54.50 tons in 2019 (Kementrian kesehatan republik indonesia, 2020). The high rate of domestic consumption makes farmers cultivate with a stocking system or intensification to achieve the target of catfish production. Intensification is an alternative to increase fishery commodity production based on increasing stocking density in limited land. The increase in stocking density has an unfavorable impact on sustainability in the form of a decrease in the cultivation environment quality (Hermawan et al., 2014). Environmental quality degradation is caused by organic waste from leftover feed and manure, where toxic inorganic nitrogen compounds generally dominate the waste. The high use of artificial high-protein feed in intensive aquaculture farming caused environmental pollution and provided disease opportunities (Asaduzzaman et al., 2008; De Schryver et al., 2008)). Diseases in catfish are caused by parasites, viruses, and bacteria (Sari et al., 2020; Sarjito et al., 2013).

One of the bacteria that has been isolated from catfish is the bacterium Aeromonas hydrophila

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(Indrawati et al., 2020; Wulandari et al., 2019). A. hydrophila is a microorganism that causes Motile Aeromonas Septicemia (MAS) disease. It is also known as hemorrhage septicemia with a mortality rate of 100%, resulting in significant losses (Yogananth et al., 2019; Anggraini et al., 2016; Lukistyowati & Kurniasih, 2012). A. hydrophila is a pathogen for freshwater fish and marine fish commodities (Hazrat Ali et al., 2014). The presence of this disease results in antibiotic compounds that are used as prevention. Continuous use of antibiotics can cause pathogenic bacteria to become resistant. Besides, it is also possible to have antibiotic residues in the fish's body, making it dangerous if consumed. The various antibiotics used on cultivates in the healing process can inappropriately harm the cultivated cultivates (Sarjito et al., 2020; Yuhana et al., 2008). The continuous use of antibiotics and chemicals can lead to bacterial resistance to antibiotics, damage the aquatic environment, and poison fish so that antibiotics become ineffective (Juanda & Jayadi, 2018; Alfiyanti et al., 2019). Resistant bacteria are expected to increase from 700,000 deaths globally in 2014 to more than ten million by 2050 (O'Neill, 2016).

The rapid development of fish farming is related to the large number of consumer demands, which has a consequence on the high use of antibiotic compounds. The use of antibiotic compounds is related to the presence of pathogenic A. hydrophila in the cultivation area. These antibiotics are used to prevent and treat disease to prevent losses due to bacterial infections, but this causes the emergence of resistance in bacteria (de Melo et al., 2011). The nature of antibiotic resistance can be transferred through a horizontal gene transfer mechanism to other bacteria, including pathogenic bacteria, harming human health. It is necessary to research the nature of bacterial resistance and antibiotic-resistant genes in catfish culture in Muara Jambi, Bungo, and Merangin districts, which are the largest catfish cultivation centers in Jambi province.

This bacteria is a pathogen, both in humans and animals, especially fish (Pratama et al., 2022). Some Gram-negative bacteria do not release toxic fluids, but instead produce endotoxins which are released when cells die or rupture. Endotoxin is a lipopolysaccharide in bacterial cell walls (Ho et al., 2023).

Method

This study used 18 isolates of A. hydrophila, archival isolates from catfish in aquaculture in the Muara Jambi, Bungo, and Merangin Regencies, Jambi Province. The research was conducted at the Integrated Laboratory of the Medical Microbiology Division of the Department of Animal Disease and Public Health, IPB University.

Antibiotics Susceptibility Test

Antibiotic susceptibility testing was carried out by the Kirby Bauer disk diffusion method using Mueller-Hinton agar based on the Clinical and Laboratory Standards Institute guidelines (Wayne, 2006). The antibiotics used in this study included tetracycline (TE) 30 µg, oxytetracycline (OT) 30 µg, ampicillin (AMP) 10 µg, amoxicillin, ciprofloxacin, enrofloxacin (EN) 5 µg, and streptomycin. The bacterial suspension used came from a bacterial colony diluted to a McFarland standard of 0.5 or equivalent to 1.5 × 108 CFU/ml, 1 ml of the suspension was poured then flattened on Mueller-Hinton agar. A diffusion disc containing antibiotics was placed on Mueller-Hinton agar using sterile tweezers at the same distance and then incubated at 35 °C for 16–18 hours. The antibiotic inhibition zone is measured and interpreted based on the 2018 CLSI Standard, which consists of categories including susceptible (S), intermediate (I), and resistant (R). Categories are determined from the range of the antibiotic inhibition zone's diameter formed on Mueller-Hinton agar (Himedialab, 2018).

DNA Extraction, Amplification, and Visualization of Resistance Gene

DNA extraction from A. hydrophila was carried out using the boiling method. The 1–2 loop bacterial colony was added with 100 µl of PBS and homogenized using a vortex, then heated at 100 ° C for 10 minutes. The heated suspension is cooled for 4 minutes, centrifuged for 2 minutes at a speed of 12500 rpm to precipitate the bacterial cells. Furthermore, 50 µl of the supernatant DNA was taken and put into a 1.5 ml microtube. The DNA results will be used for gene detection by PCR (Qabajah et al., 2014; Indrawati et al., 2020). The total volume of the reaction was 25 µl, consisting of 2 µl of template DNA, 12.5 µl of PCR mix, 1.6 µl of 10 µM forward primers, 1.6 µl of 10 µM of reverse primers, and 7.3 µl of dH_2O. The amplification process begins with pre-denaturation at 95 ° C for 3 minutes and denaturation at 95 ° C for 30 seconds. The following process is annealing at a temperature suitable for the primer used (Table 1), extension at 72 °C for 1 minute, and amplification followed by a final extension at 72 °C for 5 minutes. The amplification process is 35 cycles. The amplified samples were then visualized by electrophoresis using 1.5% agarose gel and staining with 0.5 µg /ml ethidium bromide. The marker used is 100 bp as the standard measure.

Table 1. Primers Used to Detect the Antibiotic Resistance Gene

Antibiotics	Gene	Base sequence
Ampicillin	ampC[a]	(F)5´-AATGGGTTTTCTACGGTCCTG-3´
		(R)5´-GGGCAGCAAATGTGGAGCAA-3´
Quinolone	qnr(A)	(F)5´-CCTGCGAGTACAAACTGG-3´
		(R)5´-TCAAGGTAACCAGCCAAC-3´
	qnr(B)	(F) 5´-GATCGTGAAAGCCAGAAAGG-3´
		(R) 5´-ACGATGCCTGGTAGTTGTCC-3´
	qnr(S)	(F) 5´-GATCGTGAAAGCCAGAAAGG-3´
		(R) 5´-ACGATGCCTGGTAGTTGTCC-3´

Result and Discussion

The sensitivity test of A. hydrophila bacteria to antibiotics was carried out by measuring the bacterial growth inhibition zone around the antibiotic disk. The zone of inhibition is measured and interpreted according to the 2018 CLSI standard (Figure 1). The results of the susceptibility test to antibiotics showed that all samples were 100% resistant to tetracycline, 77.7% resistant to ampicillin, 77.7% resistant to amoxicillin, and 76.2% resistant to enrofloxacin. All samples were 100% sensitive to Enrofloxacin and Streptomycin (Table 2).

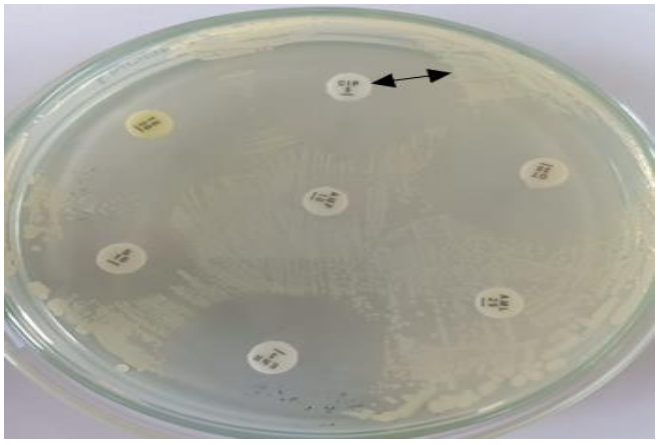

Figure 1. Measurement of inhibition zone

Table 2. The Result of Antibiotic Susceptibility Test in A. hydrophila (n=18)

Antibiotics	Sensitive		Intermediate		Resistance	
	n	%	n	%	n	%
Tetracycline	0	0%	0	0%	18	100%
Oxytetracycline	1	5.5%	0	0%	17	94.4%
Ampicillin	2	11.1%	2	11.1%	14	77.7%
Amoxycillin	1	5.5%	3	16.6%	14	77.7%
Ciprofloxacin	15	83.3%	0	0%	3	16.6%
Enrofloxacin	18	100%	0	0%	0	0%
Streptomycin	18	100%	0	0%	0	0%

The high resistance of antibiotics to amoxicillin, ampicillin, and penicillin was shown in Aeromonas strains isolated from pond water in Poland (Stratev et al., 2015; Zdanowicz et al., 2020). Aeromonas strains isolated from goldfish and rainbow trout pond water were resistant to clindamycin with a percentage of 96% (Mudryk et al., 2015). The A. hydrophila strain tested for resistance to chloramphenicol and ciprofloxacin showed a 5-6% percentage of resistance to these antibiotics (Zdanowicz et al., 2020). Aeromonas strains are resistant to the lincosamide antibiotic class, namely lincomycin, pirlimycin, and clindamycin (Ko et al., 2003; Orozova et al., 2008; Yu et al., 2015). According to Carvalho et al. (2012), samples isolated from Portuguese waters showed resistance to amoxicillin, with 59%, 90% to cefalotin, and 80% to ticarcillin.

Identification of antibiotic resistance genes is needed to determine the presence of genes encoding an antibiotic's resistance. Molecular detection of resistance genes was carried out against A. hydrophila isolates categorized as resistant. In this study, three genes were used: ampC, qnrA, qnrB, and qnrS genes (Table 3).

Table 3. The Number and Percentage of Antibiotic Resistance Gene Isolated from A. hydrophila

Antibiotics	Number of the resistant strain	Resistance gene	Number of resistance gene	Percentage of resistance gene (%)
Beta lactam	14	ampC	8	57.14
quinolone	4	qnrA	3	75
		qnrB	1	25
		qnrS	3	75

AmpC gene is one of the genes encoding the antibiotic resistance to beta-lactam, and this study found nine isolates of A. hydrophila ampC resistant gene with 191 bp amplicon (Figure 2). The presence of the ampC gene in bacterial cells is related to resistance to ampicillin. According to Adesiji et al. (2014), ampicillin is a beta-lactam antibiotic. The beta-lactam group's resistance mechanism is the formation of an enzyme that destroys penicillin, namely the beta-lactamase enzyme. This enzyme causes the betalactam ring's opening in penicillins and cephalosporins to impairs antimicrobial activity, both in Gram-positive and Gram-negative bacteria.

According to Zdanowicz et al. (2020), ampicillin, amoxicillin, penicillin, and clindamycin are β-lactam class antibiotics often used humans medicine, veterinary medicine, and aquaculture. The percentage results show that 96-99% of the β-lactam group is used to prevent Aeromonas bacterial infection in aquaculture. Research conducted by Yu et al. (2015) found that A. hydrophila strains with a percentage of 70-100% were resistant to β-lactam antibiotics, namely ampicillin, amoxicillin, and

penicillin from goldfish aquaculture in Korea. Yano et al. (2015) stated that β-lactam class antibiotics are widely used in Bangkok's goldfish ponds. A. hydrophila is an organism capable of transferring antibiotic resistance by genetic agents, namely plasmids, transposons, IS elements, Integron class I (Dar & Kamili, 2016; Patil et al., 2016; Piotrowska & Popowska, 2014). According to Hardi (2018), resistance to antibacterials is influenced by the chromosome arrangement of bacteria. Aeromonas bacteria have β-lactamases in plasmids or integrons, which can inhibit β-lactam produced by microbes. According to Saavedra et al. (2004), genus Aeromonas is considered naturally resistant to β-lactam antibiotics because the β-lactam structure is chemically unstable β-lactam chromosomes easily hydrolyze it.

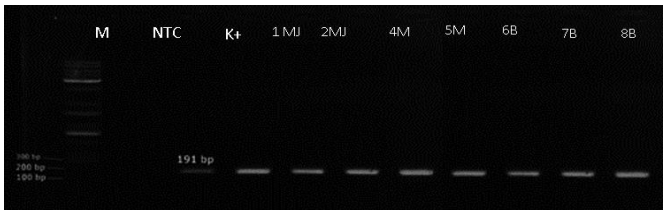

Figure 2. The amplification results of the ampC gene (191 bp) encoding ampicillin and amoxicillin resistance in samples of Aeromonas hydrophila. M (Marker 100 bp), K + (Positive control), NTC (Non template control), 1MJ (Muaro Jambi), 2MJ (Muaro Jambi), 4M (Merangin), 5M (Merangin), 6B (Bungo), 7B (Bungo), 8B (Bungo), 9MJ (Muaro Jambi)

The qnrA, qnrB, and qnrS genes were also found in this study (Figure 3). The A. hydrophila strain isolated from wastewater was resistant to four antibiotics: quinolones, aminoglycosides, β-lactams, and tetracyclines (Piotrowska & Popowska, 2014). Research conducted by Figueira et al. (2011) stated that the isolation from wastewater most of the quinolone resistance samples resulted from chromosome mutations. Mutations in the gyrA and parC genes, which are genetic determinants of quinolone resistance. The majority of these strains were resistant to nalidixic acid and included in these strains A. hydrophila and A. caviae. The high frequency of mutations occurs due to contact with mutagenic substances with high wastewater concentrations (Miyahara et al., 2011). There are two plasmid-mediated quinolone resistance genes found only in A. hydrophila, qnrS, and accA-cr (Janda & Abbott, 2010).

Conclusion

Isolation of bacteria from catfish obtained from catfish ponds in Jambi was resistant to antibiotics and resistance genes were found in isolates that were resistant to antibiotics, namely A. hydrophila was resistant to tetracycline (100%), oxytetracycline (94.4%), ampicillin (77.7 %), and amoxicillin (77.7%) and still sensitive to ciprofloxacin (83.3%), enrofloxacin (100%), and streptomycin (100%).

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Author Contributions
Conceptualization; T. W.; methodology.; T. W.; eesources.; N.S.P.; writing-original draft preparation result.; T.W.A.; writing—review and editing.; A.I.; All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest
The authors declare that there is no conflict of interest regarding the publication of this paper.

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