

# Antibiotic resistance of *Aeromonas* spp. isolated from diseased walking catfish (*Clarias* sp.)

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**Abstract.** Mulia DS, Isnansetyo A, Pratiwi R, Asmara W. 2021. Antibiotic resistance of *Aeromonas* spp. isolated from diseased walking catfish (*Clarias* sp.). *Biodiversitas* 22: 4839-4846. *Aeromonas* spp. is known to be pathogenic to freshwater fish, including catfish. Antibiotics are often used to overcome bacterial attacks. However, the indiscriminate use of antibiotics, especially at incorrect doses and frequencies, could result in the emergence of resistant bacteria. The presence of resistance genes is also suspected to trigger the resistance potential of *Aeromonas* sp. against antibiotics. The objective of this study is to characterize the resistance genes of *Aeromonas* spp. isolated from diseased walking catfish (*Clarias* sp.). Ten *Aeromonas* spp. were isolated from infected walking catfish cultivated in Java. The resistance genes assessed included *tetA*, *strA-strB*, and *qnrA*. The antibiotics tested consisted of ampicillin (AMP), erythromycin (ERY), oxytetracycline (OXT), kanamycin (KAN), enrofloxacin (ENRO), and chloramphenicol (CHL). Results showed that some *Aeromonas* spp. had at least one out of the three genes assessed (i.e., *tetA* and *strA-strB*) but none had the *qnrA* gene. *Aeromonas* spp. were sensitive to KAN, ENRO, and CHL but resistant to AMP, ERY, and OXT. Thus, KAN, ENRO, and CHL are effective antibiotics for treating fish infected with *Aeromonas* spp.

**Keywords:** *Aeromonas*, antibiotics, walking catfish, gene, resistance

## INTRODUCTION

Several obstacles, especially disease, must be addressed for successful freshwater fish cultivation. The diseases cause high mortality rates in fish, which can result in crop failure and massive financial losses. This problem is prevalent even in walking catfish (*Clarias* sp.) cultivation. Motile *Aeromonas* septicemia disease or aeromoniasis is caused by *Aeromonas* spp. These pathogens are opportunistic, gram negative, rod-shaped, facultative anaerobic bacteria, oxidase-positive, motile or non-motile, and non-spore-forming (Erdem et al. 2011; Parker and Shaw 2011; Aravena-Román et al. 2013; Percival and Williams 2014; Martínez-Murcia et al. 2016; Stratev and Odeyemi 2016).

*Aeromonas* spp. can attack nearly all types of fish and aquatic biota. Previous studies reported that *Aeromonas* spp. attacks catfish (*Clarias gariepinus*), Nile tilapia (*Oreochromis niloticus*), Arapaima gigas, eel (*Anguilla japonica*), rainbow trout (*Onchorynchus mykiss*), shrimp (*Litopenaeus vannamei*, *Penaeus monodon*), Chinese giant salamander (*Andrias davidianus*), and turtle (*Ocadia sinensis*, *Pseudemys peninsularis*) (Wang et al. 2012; Vega-Sanchez et al. 2014; Yano et al. 2015; Dias et al. 2016; Guo et al. 2016; Hassan et al. 2017; Wimalasena et al. 2017; Sellegounder et al. 2018; Eid et al. 2019). In Indonesia, the first *Aeromonas* spp. outbreak was recorded in West Java in 1980. *Aeromonas* spp. attack caused the

death of 82.2 tons of fish within 1 month. Another attack was recorded in Central Java in 1984, in which approximately 1.6 tons of catfish died (Angka 2001). Since then, *Aeromonas* spp. infection in fish has become increasingly common. Besides its virulence potential, the ability of *Aeromonas* spp. to cause disease in fish may be attributed to its resistance to antibiotics; this resistance enables the bacteria to survive, become immune to antibiotics, and continue infecting fish and other aquatic biotas. *Aeromonas* spp. are well known for their resistance genes and natural defense system. Despite the potential dangers of *Aeromonas* spp. infection, there is a lack of research concerning this important pathogen in Indonesia. Research abroad has successfully detected various resistance genes in *Aeromonas* spp., including *tetE*, *tetO*, *tetA*, *bla<sub>TEM</sub>*, *strA-strB*, *qnrS2*, and *qnrA* (Nawaz et al. 2012; Deng et al. 2014; Varela et al. 2016; Harnisz and Korzeniewska 2018; De Silva et al. 2020). Knowledge of the resistance genes of this bacterium is essential to determine the most suitable antibiotics for controlling *Aeromonas* spp. infection.

Several antibiotics, including chlortetracycline, oxytetracycline (OXT), tetracycline, erythromycin (ERY), and enrofloxacin (ENRO) (Kepmen-KP 2014), as well as ampicillin (AMP), kanamycin (KAN), and chloramphenicol (CHL), are often used to control virulent bacteria. However, the inappropriate use of antibiotics, especially in terms of time, dose, and target, is common.

This practice may potentially lead to bacterial resistance (Parker and Shaw 2011). Research on antibiotics is also necessary to identify effective drugs against specific pathogens. The resistance of *Aeromonas* spp. to various antimicrobials is feature that must be comprehensively studied. Thus, the aim of the present study is to characterize the resistance of *Aeromonas* spp. isolated from walking catfish (*Clarias* sp.).

## MATERIALS AND METHODS

### Bacterial isolates of *Aeromonas* spp.

Bacterial strains of *Aeromonas* spp. were obtained from the kidneys of infected walking catfish cultivated ponds in Yogyakarta, Central Java, and West Java, Indonesia.

### Detection of resistance genes in *Aeromonas* spp.

The resistance genes investigated in this work included *tetA*, *strA-strB*, and *qnrA*. Polymerase chain reaction (PCR) was conducted using tubes with a capacity of 200  $\mu$ L. Each 25  $\mu$ L reaction mixture consisted of 12  $\mu$ L of 2 $\times$  PCR Master Mix (Mytaq HS Red Mix, Bioline), 1  $\mu$ L each of the forward and reverse primers, 1  $\mu$ L of the DNA template (20 ng), and 10  $\mu$ L of NFW (Mulia et al. 2020). The PCR products were electrophoresed with 1.5% gel agarose gel running with tris acetate EDTA (TAE) buffer.

### Resistance test of *Aeromonas* spp. by minimum inhibitor concentration (MIC) of antibiotics

*Aeromonas* spp. resistance tests were conducted on bacterial isolates according to the MICs of the selected antibiotics, via the microdilution method (NCCLS 1994). Six antibiotics, namely oxytetracycline (OXT), kanamycin (KAN), ampicillin (AMP), enrofloxacin (ENRO), erythromycin (ERY), chloramphenicol (CHL) was used for resistance test. MIC test was performed in triplicates. The antibiotics tested included AMP, KAN (Wako Pure Chemical Industries, Osaka, Japan), OXT, ENRO, ERY, and CHL (Sigma Corporate, St. Louis, MI, USA). Bacterial isolates were inoculated on Mueller–Hilton broth. Antimicrobial susceptibility assay was performed in plates with flat-bottomed wells and then plates were incubated at 30°C for 24 h. After incubation, 10  $\mu$ L of resazurin was added to all wells of the microplate. Another cycle of incubation was performed at 30°C, and observation was conducted 1 h later.

### Resistance development test of *Aeromonas* spp. by minimum bactericidal concentration (MBC) of antibiotics

MBCs were determined from the obtained MICs, and the effects of MBCs of MIC, 2 $\times$ MIC, 4 $\times$ MIC, and 6 $\times$ MIC (Isnansetyo and Kamei 2009) were also determined. The MBC test was conducted using the microdilution method (NCCLS 1994). After 24 h of incubation at 30°C, bacteria treated with the selected concentrations of antibiotics, including control, were grown on glutamate starch phenyl (GSP) medium by using the streak method. Each experiment was performed in triplicate. Samples grown in GSP medium were then incubated at 30°C for 48 h. Colonies appeared on the streaked line in GSP medium indicating bacterial growth. Bactericidal effects were identified when bacteria from the wells containing antibiotics failed to grow after 48 h of incubation.

### Data analysis

Data from the detection of resistance genes, resistance tests of *Aeromonas* spp. based on MICs, and resistance development tests based on MBCs were analyzed descriptively. The primers of the resistance genes (i.e., *tetA*, *strA-strB*, and *qnrA*) were used according to Mulia et al. (2020).

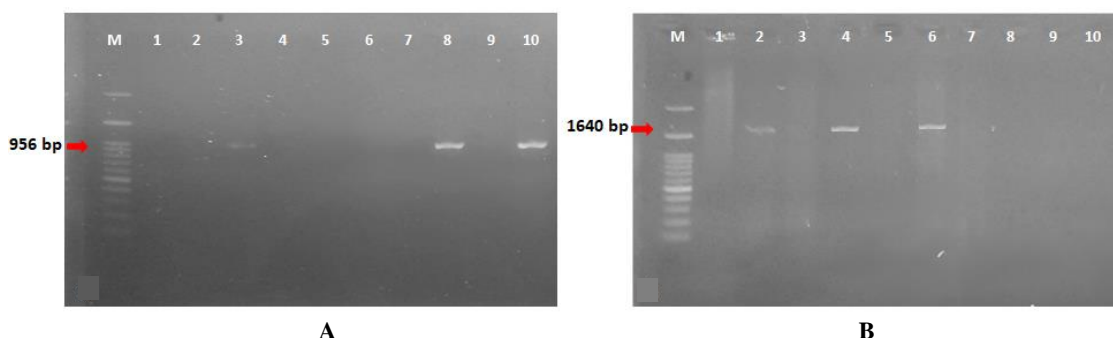
## RESULTS AND DISCUSSION

### Isolates of *Aeromonas* spp.

Ten isolates of *Aeromonas* spp., including four *A. dhakensis* isolates (SB-01, MS-03, KK-02, and KO-01), four *A. veronii* bv. *veronii* isolates (SC-03, DW-04, BCp-02-1, and BCp-02-2), one *A. caviae* isolate (MD-03), and one *A. hydrophila* isolate (BCp-01-2) were assessed in this study (Table 1).

### Detection of resistance genes of *Aeromonas* spp.

The result showed that *tetA* gene was detected in three isolates, namely, *A. dhakensis* MS-03, *A. hydrophila* BCp-01-2, and *A. veronii* bv. *veronii* BCp-02-2 (Figure 1a). The *strA-strB* gene was found in three isolates, namely, *A. veronii* bv. *veronii* SC-03, *A. dhakensis* KK02, and *A. caviae* MD-03 (Figure 1b). The *qnrA* gene was not detected in any of the isolates.



**Figure 1.** Amplification of resistance genes in *Aeromonas* spp. (a) *tetA* gene, 956 bp; M, DNA Marker 100 bp; Lane 1, SB-01; 2, SC-03; 3, MS-03; 4, KK-02; 5, KO-01; 6, MD-03; 7, DW-04; 8, BCp-01-2; 9, BCp-02-1; 10, BCp-02-2. (b) *strA-strB* gene, 1640 bp.

Table 1 shows that *tetA* genes was found in *A. dhakensis* MS-03, *A. hydrophila* BCp-01-2, and *A. veronii* bv. *veronii* BCp-02-2 isolates (30% isolates); *strA-strB* genes was found in *A. veronii* bv. *veronii* SC-03, *A. dhakensis* KK02, and *A. caviae* MD-03 isolates (30% isolates), and *qnrA* gene were not found in any isolate. The *qnrA* gene, as in previous studies, was not detected in any of the isolates tested (Varela et al. 2016; Wimalasena et al. 2017).

Six isolates showed one resistance gene, whereas four isolates did not show a single resistance genes. Two *A. dhakensis* isolates, i.e. MS-03 and KK-02, had *tetA* and *strA-strB* resistance genes, respectively. However, two other *A. dhakensis* isolates, i.e. SB-01 and KO-01, had no resistance genes. One *A. veronii* bv. *veronii* isolate, i.e. BCp-02-2, had *tetA* gene, while SC-03 had *strA-strB* gene. No resistance genes were detected in DW-04 and BCp-02-1. Previous research results reported that the *tetA* gene is found in *A. veronii* isolated from fish and turtles (Deng et al. 2014). The *strA-strB* gene was detected in *A. caviae* MD-03 but *tetA* gene was not. These results differ from previous studies, which reported the detection of *tetA* gene, but not *strA* or *strB* genes in *A. caviae* isolated from shrimp and catfish (Deng et al. 2014; Mulia et al. 2020).

*A. hydrophila* BCp-01-1 was detected to have *tetA* gene but not *strA-strB* gene. The same finding was reported by Deng et al. (2014), who detected *tetA* gene but not *strA-strB* gene in *A. hydrophila* isolated from fish. Other studies have successfully detected *tetA* gene from *A. hydrophila* isolated from shrimp and farm-raised fish (De Silva et al. 2018). However, different results were obtained by Tipmongkolsilp et al. (2012), who did not find *tetA* gene in *A. hydrophila*.

The results revealed that only *tetA* and *strA-strB* genes were found in the selected isolates, but these genes were not always present in every isolate. However, the presence

or absence of *tetA* and *strA-strB* genes in isolates did not always translate to consistent resistance to the tested antibiotics. In fact, the presence of a resistance gene in a bacterium indicates some protection against the effects of antibiotics. The *tetA* gene encodes proteins that protect ribosomes and suppress tetracycline. These genes are often associated with plasmids, transposons, and integrons as conjugative and mobilization elements in bacteria. Most of the *tet* genes encode transport proteins. The proteins are responsible for pumping antibiotics out of bacterial cells and maintaining low intercellular antibiotic concentrations to allow the normal function of ribosomes (Nguyen et al. 2014).

The *strA-strB* gene is an aminoglycoside-resistant gene. Aminocyclitol is an aminoglycoside that inhibits protein synthesis to kill bacteria. This antibiotic group binds to the 16S rRNA gene and disrupts the integrity of the bacterial cell membrane (Shakil et al. 2008).

Previous studies have observed inconsistency between the occupation of resistance genes and resistance to antibiotics and vice versa. Out of 12 isolates of *Aeromonas* spp. that were resistant to the aminoglycoside group, only one was detected to have *strA-strB* gene (De Silva et al. 2020). In addition, *Aeromonas* spp. isolated from fish and shrimp did not have amplicons for *strA-strB* gene, but some isolates were resistant to the aminoglycoside group (Deng et al. 2014). The results of this study indicate that the presence of resistance genes varies among species and that the presence of these genes is not always synonymous with the ability to resist antibiotics (Dahanayake et al. 2019). Increasing the understanding of the diversity distribution of bacteria resistant to antibiotics is essential to prevent these bacteria from spreading (Stoll et al. 2012). The presence of a resistance gene does not consistently mean resistance to antibiotics and vice versa (Dahanayake et al. 2019).

**Table 1.** *Aeromonas* spp. with resistance genes

Isolates	Origin of isolates	Species	Resistance genes detected in <i>Aeromonas</i> spp.			Total (%)
			<i>tetA</i>	<i>strA-strB</i>	<i>qnrA</i>	
SB-01	Sleman (DIY)	<i>A. dhakensis</i> strain P21	-	-	-	0 (0%)
SC-03	Sleman (DIY)	<i>A. veronii</i> bv. <i>veronii</i> strain ATCC 35624	-	+	-	1 (33.33%)
MS-03	Magelang (Central Java)	<i>A. dhakensis</i> strain P21	+	-	-	1 (33.33%)
KK-02	Kulonprogo (DIY)	<i>A. dhakensis</i> strain P21	-	+	-	1 (33.33%)
KO-01	Kedung Ombo (Central Java)	<i>A. dhakensis</i> strain P21	-	-	-	0 (0%)
MD-03	Magelang (Central Java)	<i>A. caviae</i> strain ATCC 15468	-	+	-	1 (33.33%)
DW-04	Demak (Central Java)	<i>A. veronii</i> bv. <i>veronii</i> strain ATCC 35624	-	-	-	0 (0%)
BCp-01-2	Bogor (West Java)	<i>A. hydrophila</i> strain ATCC 7966	+	-	-	1 (33.33%)
BCp-02-1	Bogor (West Java)	<i>A. veronii</i> bv. <i>veronii</i> strain ATCC 35624	-	-	-	0 (0%)
BCp-02-2	Bogor (West Java)	<i>A. veronii</i> bv. <i>veronii</i> strain ATCC 35624	+	-	-	1 (33.33%)
Total (%)			3 (30%)	3 (30%)	0 (0%)	6

### Resistance test of *Aeromonas* spp. based on the MICs of antibiotics

The resistance of *Aeromonas* spp. was determined via the microplate microdilution method on the basis of the MICs of the antibiotics. Bacteria that grew in wells with the highest concentration of antibiotics (10 µg/mL) were considered resistant to these antibiotics. Ambadiang et al. (2020) revealed that MIC ≤ 10 µg/mL indicates strong activity. Thus, no inhibitory test with antibiotic concentrations above 10 µg/mL was conducted because it is no longer effective. The results showed that *A. dhakensis* isolates SB-01, MS-03, KK-02, and KO-01 were resistant and sensitive to various levels of antibiotics (Table 2). Isolate SB-01 was resistant to ERY, but sensitive to OXT and AMP (10 µg/mL), KAN and CHL (5 µg/mL), and ENRO (0.2 µg/mL). Isolate MS-03 was resistant to KAN, AMP, and ERY, but sensitive to CHL (7.5 µg/mL), OXT (2.5 µg/mL), and ENRO (1.875 µg/mL). Isolate KK-02 was unaffected by OXT and AMP, but sensitive to KAN (7.5 µg/mL), ERY (3.75 µg/mL), CHL (2.5 µg/mL), and ENRO (0.1 µg/mL). Isolate KO-01 was unaffected by AMP, ERY, and CHL, but sensitive to KAN (2.5 µg/mL), OXT (1.25 µg/mL), and ENRO (0.313 µg/mL).

*Aeromonas veronii* bv. *veronii* SC-03, DW-04, BCp-02-1, and BCp-02-2 were resistant and sensitive to different levels of antibiotics. Isolate SC-03 was insensitive to OXT, AMP, ERY, and CHL, but sensitive to KAN (2.5 µg/mL) and ENRO (0.05 µg/mL). Isolate DW-04 was resistant to ERY, but sensitive to OXT (10 µg/mL), CHL (1.875 µg/mL), KAN and ENRO (0.625 µg/mL), and AMP (0.2 µg/mL). Isolate BCp-02-1 was resistant to AMP, but sensitive to OXT and KAN (5 µg/mL), ERY (0.625 µg/mL), CHL (0.313 µg/mL), and ENRO (0.025 µg/mL). Isolate BCp-02-2 was resistant to OXT and AMP, but sensitive to ERY (3.75 µg/mL), KAN (2.5 µg/mL), CHL (1.25 µg/mL), and ENRO (0.2 µg/mL). *A. caviae* MD-03 was unaffected by OXT, AMP, ERY, and CHL, but sensitive to KAN (2.5 µg/mL) and ENRO (0.05 µg/mL). *A. hydrophila* BCp-01-2 was resistant to AMP and ERY, but sensitive to OXT (10 µg/mL), KAN (6.25 µg/mL), CHL (1.875 µg/mL), and ENRO (0.013 µg/mL).

The resistance of *Aeromonas* spp. against the tested antibiotics tended to vary. However, previous reports revealed increased bacterial resistance against most commonly used antibiotics. The results showed variations in resistance among each of the *A. dhakensis* and *A. veronii* bv. *veronii* isolates. Most of the isolates were resistant to AMP, ERY, and OXT, some isolates were resistant to KAN and CHL. All the isolates showed sensitivity to ENRO in the resistance test.

*A. dhakensis* SB-01 and KO-01, as well as *A. veronii* bv. *veronii* DW-04 and BCp-02-1, did not have *tetA* and *strA-strB* genes and were found to be sensitive to OXT and KAN. *A. dhakensis* MS-03, *A. hydrophila* BCp-01-2, and *A. veronii* bv. *veronii* BCp-02-2 had only *tetA* gene. *A. veronii* bv. *veronii* BCp-02-2 was resistant to OXT, but the other two isolates were actually sensitive to OXT. The absence of *strA-strB* gene rendered *A. hydrophila* BCp-01-

2 and *A. veronii* bv. *veronii* BCp-02-2 sensitive to KAN, but *A. dhakensis* MS-03 was resistant to this antibiotic. *A. veronii* bv. *veronii* SC-03 and *A. dhakensis* KK-02 had *strA-strB* genes and were resistant to OXT and sensitive to KAN. *A. caviae* MD-03 had only *strA-strB* gene and was resistant to OXT but sensitive to KAN. *A. hydrophila* BCp-01-2 had only *tetA* gene and found sensitive to KAN and OXT. Some isolates were not detected to have *tetA* gene, but were resistant to OXT, presumably because of the presence of other *tet* genes participating in OXT resistance. However, this study did not detect these genes. Verner-Jeffreys et al. (2019) reported that most the isolates of *Aeromonas* spp. resistant to tetracyclines and had *tetA*, *tetD* or *tetE* genes. De Silva et al. (2020) reported that half (50%) of the 32 isolates of *Aeromonas* spp. tested were proven to be resistant to tetracyclines and OXTs, but *tetE* gene was detected in only two isolates and *tetA* gene was detected in only one isolate. Fauzi et al. (2021) reported that isolates of *Aeromonas* spp. that were resistant to kanamycin (the aminoglycoside group) were detected to have *strA-strB* gene.

Vega-Sanchez et al. (2014) reported that *A. hydrophila* isolated from infected and healthy rainbow trout (*O. mykiss*) in Mexico, is 100% resistant to AMP. *A. hydrophila* isolated from clinical cases (43 isolates) of several aquatic animals, including ornamental goldfish (*Carassius auratus*), turtles (*Amyda cartilaginea*), freshwater shrimp (*Macrobrachium rosenbergii*), goldfish (*Cyprinus carpio* L.), Chinese freshwater fish (*Megalobrama amblycephala*), Chinese shrimp (*Penaeus chinensis*), clams (*Mytilus edulis*), silver goldfish (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodon idellus*), and eel (*Monopterus albus*), exhibit 100% resistance to AMP, 90.70% resistance to ERY, and 2.33% resistance to ENRO (Guo et al. 2014). The same finding is reported by Stratev et al. (2013), who confirmed that *A. hydrophila* isolates are resistant to AMP and ERY.

The MICs were used to observe the resistance of *Aeromonas* spp. to antibiotics (Table 2). Each *Aeromonas* spp. strain was resistant to at least one type of antibiotic. *A. veronii* bv. *veronii* SC-03 and *A. caviae* MD-03 were resistant to four types of antibiotics but had only one of the three genes tested. This finding shows that having a resistance gene confers *Aeromonas* spp. with not only resistance but also the ability to adapt and respond to antibiotics.

Table 3 shows that eight isolates (80%) of *Aeromonas* spp. were resistant to AMP, seven isolates (70%) were resistant to ERY, four isolates (40%) were resistant to OXT, three isolates (30%) were resistant to CHL, and one isolate (10%) was resistant to KAN. All of the *Aeromonas* spp. were sensitive to ENRO. OXT, AMP, ENRO, CHL, and ERY have a broad spectrum of activity against gram-positive and negative bacteria, although ERY is only partially active against gram-negative bacteria. KAN has a spectrum of gram-negative activity (Soares et al. 2012; Eyler and Shvets 2019).

**Table 2.** Resistance of *Aeromonas* spp. based on the MICs of antibiotics

Isolates	Types of antibiotics					
	OXT (µg/mL)	KAN (µg/mL)	AMP (µg/mL)	ENRO (µg/mL)	ERY (µg/mL)	CHL (µg/mL)
SB-01	10	5	10	0.2	R	5
SC-03	R	2.5	R	0.05	R	R
MS-03	2.5	R	R	1.875	R	7.5
R	-	7.5	R	0.1	3.75	2.5
KO-01	1.25	2.5	R	0.313	R	R
MD-03	R	2.5	R	0.05	R	R
DW-04	10	0.625	0.2	0.625	R	1.875
BCp-01-2	10	6.25	R	0.013	R	1.875
BCp-02-1	5	5	R	0.025	0.625	0.313
BCp-02-2	R	2.5	R	0.2	3.75	1.25

Note: R: resistant

**Table 3.** Antibigram profiles of antibiotics tested on *Aeromonas* spp. based on MICs

Antibiotics	Number of resistant isolates (n = 10)	%	Activity spectrum	References
OXT	4	40	Gram-positive and negative	Soares et al. (2012);
KAN	1	10	Gram-negative	Eyler and Shvets (2019)
AMP	8	80	Gram-positive and negative	
ENRO	0	0	Gram-positive and negative	
ERY	7	70	Gram-positive and negative	
CHL	3	30	Gram-positive and negative	

According to the antibiogram profiles result, *Aeromonas* spp. were the most resistant to AMP (80%). All *Aeromonas* spp. isolated from sediments, bivalves, sea cucumbers, and seawater (53 isolates) showed resistance (100%) to AMP and penicillin (Odeyemi and Ahmad 2017). Igbinosa and Okoh (2012), who studied 24 isolates of *Aeromonas* spp. from wastewater treatment plants in Alice and Fort Beaufort, Eastern Cape Province, South Africa, indicated similar findings. These results reveal that all *Aeromonas* spp. isolates are resistant (100%) to AMP and penicillin. As many as 61 isolates from *A. hydrophila*, *A. veronii* bv. *veronii*, and *A. veronii* bv. *sobria* were proven to be resistant (100%) to AMP and penicillin (Simon et al. 2016). Approximately 50 *Aeromonas* spp. isolates from various livestock products showed 100% resistance to AMP (Didugu et al. 2016). This resistance to AMP is believed to be due to the frequent use of the antibiotic to treat *Aeromonas* spp., thus allowing the latter to adapt to this group of antibiotics despite AMP having a wide spectrum of activity against gram-positive and negative bacteria. *Aeromonas* spp. may produce the enzyme beta-lactamase, which can damage the structure of beta-lactam. The beta-lactam structure of the penicillin group, including AMP, inhibits bacterial cell wall synthesis (Lakshmi et al. 2014).

*Aeromonas* spp. showed resistance to ERY (70%). *Aeromonas* spp. from a wastewater treatment plant in Alice, Eastern Cape Province, South Africa, are susceptible to ERY (66.7%) (Igbinosa and Okoh 2012). The results of Simon et al. (2016) indicated that *Aeromonas* spp. are insensitive to ERY by 39.97%. The results of Olaniran et al. (2015) indicated that *Aeromonas* sp. are 58% insensitive to ERY, while Stratev et al. (2013) reported that *A.*

*hydrophila* is 100% resistant to ERY. *Aeromonas* spp. maybe resistant to ERY because the latter is more effective against gram-positive than gram-negative bacteria.

*Aeromonas* spp. exhibited resistant to OXT (40%). A study by Odeyemi and Ahmad (2017) showed that *Aeromonas* spp. are unaffected to OXT by 24.5%. *Aeromonas* spp. from a wastewater treatment plant in Fort Beaufort, Eastern Cape Province, South Africa, are resistant to tetracyclines (77.8%) (Igbinosa and Okoh 2012). Zhou et al. (2019) demonstrated that *Aeromonas* spp. isolated from extra-intestinal and intestinal infections are 18.3% resistant to tetracycline. Previous studies also reported that *Aeromonas* spp. isolated from shrimp, fish and turtle are 33.3%, 14.7% and 50% resistant to tetracycline, respectively (Deng et al. 2014).

*Aeromonas* spp. showed resistance to CHL (30%). Odeyemi and Ahmad (2017) showed that *Aeromonas* spp. are insensitive to CHL by 20.8%. Zhou et al. (2019) studied *Aeromonas* spp. isolated from extra-intestinal and intestinal infections found that bacteria are resistant to CHL by 8.7%. *Aeromonas* spp. detected from a wastewater treatment plant in Fort Beaufort, Eastern Cape Province, South Africa, are susceptible to CHL by 61.1% while those isolated from a wastewater treatment plant in Alice are susceptible to CHL by 83.3% (Igbinosa and Okoh 2012).

Only one (10%) of the 10 tested isolates, namely, *A. dhakensis* MS-03, was resistant to KAN. Odeyemi and Ahmad (2017) showed that *Aeromonas* spp. are resistant to KAN by 5.7%. Didugu et al. (2016) found contradictory results and revealed that, among 50 isolates of *Aeromonas* spp. isolated from livestock products, none were resistant to KAN, but sensitive to KAN by 94% and intermediate to KAN by 6%.

**Table 4.** Potential development of *Aeromonas* spp. resistance based on antibiotic MBCs

Antibiotics	MIC ( $\mu\text{g/mL}$ )	MBC	Resistance	Antibiotic properties
OXT	1.250	$>6\times\text{MIC}$	R	Bacteriostatic
KAN	0.625	$6\times\text{MIC}$	S	Bactericidal
AMP	0.200	$>6\times\text{MIC}$	R	Bacteriostatic
ENRO	0.013	$6\times\text{MIC}$	S	Bactericidal
ERY	0.625	$>6\times\text{MIC}$	R	Bacteriostatic
CHL	0.313	$4\times\text{MIC}$	S	Bactericidal

Note: S: sensitive, R: resistant

*Aeromonas* spp. were not found to be resistant to ENRO. Didugu et al. (2016) obtained similar findings and revealed that, among 50 *Aeromonas* spp. isolates obtained from livestock products, none are found resistant to ENRO. The results of a study on 43 *A. hydrophila* isolates isolated from clinical cases showed that only 2.23% of the isolates were resistant to ENRO (Guo et al. 2014). Differences in resistance or sensitivity may be attributed to variations in isolate sources, frequency, and types of antimicrobial agents used to treat various infections in different geographic areas (Nagar et al. 2011).

#### Development of resistance by *Aeromonas* spp. based on the MBCs of antibiotics

Six types of antibiotics were tested for resistance development based on the MBC value. The MBCs used were based on the MICs. Table 4 shows that OXT, AMP, and ERY were bacteriostatic. The three other antibiotics, namely, CHL at a concentration of  $4\times\text{MIC}$ , KAN at a concentration of  $6\times\text{MIC}$ , and ENRO at a concentration of  $6\times\text{MIC}$ , were bactericidal. These results are in accordance with the antibiogram profiles of the antibiotics (Table 3), consisting of three antibiotics with the smallest number of resistant isolates, including bactericides on the MBC results. Previous research tested the effectiveness of MC21-B (produced from the marine bacteria *Pseudoalteromonas phenolica* O-BC30T) against methicillin-resistant *Staphylococcus aureus*, and results showed that MC21-B is an effective bactericidal at a concentration of  $4\times\text{MIC}$  (Isnansetyo and Kamei 2009). Other studies tested ketapang leaf extract against *A. salmonicida*, and results indicated that the MIC and MBC of the extract are 50 and 100 mg/mL, respectively; in other words, the MBC of the extract is  $2\times\text{MIC}$  (Sumino et al. 2013).

Although OXT, AMP, and ERY were bacteriostatic, they have a wide spectrum of activity against Gram-positive and negative bacteria (Tables 3 and 4). This finding is in accordance with their classification based on their working power, as described in Permenkes No. 2406 of 2011; in particular, OXT and ERY are described as bacteriostatic agents (Kementrian Kesehatan RI 2011). However, in contrast to the results of this study, AMP is classified as bactericidal (Kementrian Kesehatan RI 2011; Tjay and Rahardja 2015). KAN is a bactericidal agent with a spectrum of activity against Gram-negative bacteria (Kementrian Kesehatan RI 2011). ENRO is a bactericidal agent with a wide spectrum of activity against Gram-positive and negative bacteria; the antibiotic contains a

quinolone group, which has bactericidal properties (Tjay and Rahardja 2015). CHL is another bactericidal agent with a wide spectrum of activity against gram-positive and negative bacteria, but it is not accordance with the classification of bacterial activity, in which CHL is bacteriostatic (Kementrian Kesehatan RI 2011; Tjay and Rahardja 2015). This finding confirms that KAN, ENRO, and CHL could effectively treat fish attacked by *Aeromonas* spp. Since *Aeromonas* spp. are Gram-negative bacteria, the use of KAN in this study is appropriate, as this antibiotic focuses on Gram-negative bacteria. Likewise, the use of ENRO and CHL, which are bactericidal and show a broad spectrum of activity, is quite effective for *Aeromonas* spp. KAN irreversibly binds to the prokaryotic ribosomes of bacteria to inhibit protein synthesis, thereby inhibiting bacterial growth (Neu and Gootz 2001). CHL can inhibit protein synthesis, attach to the 50S subunit of ribosome, and interfere with the binding of new amino acids to the peptide chain being formed. CHL mainly inhibits peptidyl transferase (Neu and Gootz 2001). ENRO belongs to the fluoroquinolone class of antibiotics that could inhibit DNA gyrase (topoisomerase II) and topoisomerase IV (topo IV), which are required by bacteria to perform DNA replication. This inhibition produces a cytotoxic effect on the target cells (Babaahmady and Khosravi 2011). Study on resistance genes, susceptibility to antibiotics, and development of resistance to *Aeromonas* spp. not yet reported in Indonesia. The findings in this study may provide important information about the resistance characteristics of *Aeromonas* spp. isolated from walking catfish (*Clarias* sp.) obtained from a cultivation pond in Java Island, Indonesia.

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#### REFERENCES

- Ambadiang MMM, Atontsa BCK, Tankeo SB, Nayim P, Wamba BEN, Bitchagno GTM, Mpetga JDS, Penlap VB, Kuete V. 2020. Bark

- extract of *Cassia sieberiana* DC. (Caesalpinaceae) displayed good antibacterial activity against MDR gram-negative phenotypes in the presence of phenylalanine-arginine  $\beta$ -naphthylamide. *BMC Compl Med Ther* 20: 342. DOI: 10.1186/s12906-020-03148-3.
- Angka SL. 2001. Characterization and pathology studies of *Aeromonas hydrophila* on African catfish (*Clarias gariepinus*). Papers Philosophy of Science. Program Pascasarjana, Insitut Pertanian Bogor. [Indonesian]
- Aravena-Román M, Beaz-Hidalgo R, Inglis TJJ, Riley TV, Martínez-Murcia AJ, Chang BJ, Figueras MJ. 2013. *Aeromonas australiensis* sp. nov., isolated from irrigation water. *Intl J Syst Evol Microbiol* 63: 2270-2276. DOI: 10.1099/ijs.0.040162-0.
- Babaahmady E, Khosravi A. 2011. Toxicology of baytril (enrofloxacin). *Afr J Pharm Pharmacol* 5 (18): 2042-2045. DOI: 10.5897/AJPP11.644.
- Dahanayake PS, Hossain S, Wickramanayake MVKS, Heo GJ. 2019. Antibiotic and heavy metal resistance genes in *Aeromonas* spp. isolated from marketed Manila Clam (*Ruditapes philippinarum*) in Korea. *J Appl Microbiol* 127 (3): 941-952. DOI: 10.1111/jam.14355.
- De Silva BCI, Hossain S, Dahanayake PS, Heo GJ. 2018. Frozen white-leg shrimp (*Litopenaeus vannamei*) in Korean markets as a source of *Aeromonas* spp. harboring antibiotic and heavy metal resistance genes. *Microb Drug Resist* 24 (10): 1587-1598. DOI: 10.1089/mdr.2018.0035.
- De Silva BCI, Hossain S, Dahanayake PS, Lee D, Wickramanayake MVKS, Heo G. 2020. Multi-drug resistant mesophilic aeromonads isolated from marketed scallops (*Patinopecten yessoensis*) harboring resistance genes. *Fish Aquat Life* 28 (1): 1-10. DOI: 10.2478/aopf-2020-0001.
- Deng YT, Wu YL, Tan AP, Huang YP, Jiang L, Xue HJ, Wang WL, Luo L, Zhao F. 2014. Analysis of antimicrobial resistance genes in *Aeromonas* spp. isolated from cultured freshwater animals in China. *Microb Drug Resist* 20 (4): 350-356. DOI: 10.1089/mdr.2013.0068.
- Dias MKR, Sampaio LS, Proietti-Junior AA, Yoshioka ETO, Rodrigues DP, Rodriguez AFR, Ribeiro RA, Faria FS, Ozório ROA, Tavares-Dias M. 2016. Lethal dose and clinical signs of *Aeromonas hydrophila* in *Arapaima gigas* (Arapaimidae), the giant fish from Amazon. *Vet Microbiol* 188: 12-15. DOI: 10.1016/j.vetmic.2016.04.001.
- Didugu H, Krishnaiah N, Rao MT, Ramanipushpa RN. 2016. Antibigram of *Aeromonas* species isolated from livestock products. *Intl J Sci Environ* 5 (2): 475-478.
- Eid HM, Soliman ZI, Hanafy AT. 2019. Antibacterial resistance of *Aeromonas* species isolated from fish and water of Manzala Lake. *Suez Canal Vet Med J* 24 (2): 217-230. DOI: 10.21608/scvmj.2019.69846.
- Erdem B, Kariptaş E, Çil E, Işık K. 2011. Biochemical identification and numerical taxonomy of *Aeromonas* spp. isolated from food samples in Turkey. *Turk J Biol* 35: 463-472. DOI: 10.3906/biy-0904-11.
- Eyler RF, Shvets K. 2019. Clinical pharmacology of antibiotics. *Clin J Am Soc Nephrol* 14 (7): 1080-1090. DOI: 10.2215/CJN.08140718.
- Fauzi NNFM, Hamdan RH, Mohamed M, Ismail A, Zin AAM, Mohamad NFA. 2021. Prevalence, antibiotic susceptibility, and presence of drug resistance genes in *Aeromonas* spp. isolated from freshwater fish in Kelantan and Terengganu states, Malaysia. *Vet World* 14: 2064-2072. DOI: 10.14202/vetworld.2021.2064-2072.
- Guo P, Wang N, Liu Y, Lu C. 2014. Antimicrobial susceptibility and characterization of outer membrane proteins of *Aeromonas hydrophila* isolated in China. *J Integr Agric* 13 (4): 911-917. DOI: 10.1016/S2095-3119(13)60312-3.
- Guo SL, Yang QH, Feng JJ, Duan LH, Zhao JP. 2016. Phylogenetic analysis of the pathogenic genus *Aeromonas* spp. isolated diseased eels in China. *Microb Pathol* 101: 12-23. DOI: 10.1016/j.micpath.2016.10.016.
- Harnisz M, Korzeniewska E. 2018. The prevalence of multidrug-resistant *Aeromonas* spp. in the municipal wastewater system and their dissemination in the environment. *Sci Total Environ* 626: 377-383. DOI: 10.1016/j.scitotenv.2018.01.100.
- Hassan MA, Noureldin EA, Mahmoud MA, Fita NA. 2017. Molecular identification and epizootiology of *Aeromonas veronii* infection among farmed *Oreochromis niloticus* in Eastern Province, KSA. *Egypt J Aquat Res* 43 (2): 161-167. DOI: 10.1016/j.ejar.2017.06.001.
- Igbinosa IH, Okoh AI. 2012. Antibiotic susceptibility profile of *Aeromonas* species isolated from wastewater treatment plant. *The Sci World J* 2012: 764563. DOI: 10.1100/2012/764563.
- Isnansetyo A, Kamei Y. 2009. Anti-methicillin-resistant *Staphylococcus aureus* (MRSA) activity of MC21-B, an antibacterial compound produced by the marine bacterium *Pseudoalteromonas phenolica* O-BC30<sup>T</sup>. *Intl J Antimicrob Agents* 34 (2): 131-135. DOI: 10.1016/j.ijantimicag.2009.02.009.
- Kementerian Kesehatan RI. 2011. General Guidelines for Use of Antibiotics. Jakarta: Departmen Kesehatan RI. 63 hal. [Indonesian].
- Kepmen KP. 2014. Decree of the Minister of Marine Affairs and Fisheries RI No.52/KEPMEN-KP/2014 Concerning the Classification of Fish Medicines. Minister of Marine Affairs and Fisheries of the Republic of Indonesia, Jakarta. [Indonesian].
- Lakshmi R, Nusrin KS, Georgy SA, Sreelakshmi KS. 2014. Role of beta lactamases in antibiotic resistance: A review. *Intl Res J Pharm* 5 (2): 37-40. DOI: 10.7897/2230-8407.050207.
- Martínez-Murcia A, Beaz-Hidalgo R, Navarro A, Carvalho MJ, Aravena-Román M, Correia A, Figueras MJ, Saavedra MJ. 2016. *Aeromonas lusitana* sp. nov., isolated from untreated water and vegetables. *Curr Microbiol* 72: 795-803. DOI: 10.1007/s00284-016-0997-9.
- Mulia DS, Isnansetyo A, Pratiwi R, Asmara W. 2020. Molecular characterizations of *Aeromonas caviae* isolated from catfish (*Clarias sp.*). *AACL Bioflux* 13 (5): 2717-2732. <https://www.cabdirect.org/cabdirect/abstract/20203600153>
- Nagar V, Shashidhar R, Bandekar JR. 2011. Prevalence, characterization, and antimicrobial resistance of *Aeromonas* strains from various retail food products in Mumbai, India. *J Food Sci* 76 (7): M486-M492. DOI: 10.1111/j.1750-3841.2011.02303.x.
- Nawaz M, Khan SA, Tran Q, Sung K, Khan A.A, Adamu I, Steele RS. 2012. Isolation and characterization of multidrug-resistant *Klebsiella* spp. isolated from shrimp imported from Thailand. *Intl J Food Microbiol* 155 (3): 179-184. DOI: 10.1016/j.ijfoodmicro.2012.02.002.
- NCCLS. 1994. Performance standards for antimicrobial disk and dilution susceptibility tests for bacterial isolated from animals: Proposed standard. NCCLS Document m31-P: 1-29. 14(20).
- Neu HC, Gootz TD. 2001. Antimicrobial chemotherapy. In: Baron S (eds). *Medical Microbiology*. 5th edition. The University of Texas Medical Branch, Galvestone.
- Nguyen F, Starosta AL, Arenz S, Sohmen D, Dönhöfer A, Wilson DN. 2014. Tetracycline antibiotics and resistance mechanisms. *Biol Chem* 395 (5): 559-575. DOI: 10.1515/hsz-2013-0292.
- Odeyemi OA, Ahmad A. 2017. Antibiotic resistance profiling and phenotyping of *Aeromonas* species isolated from aquatic sources. *Saudi J Biol Sci* 24 (1): 65-70. DOI: 10.1016/j.sjbs.2015.09.016.
- Olaniran AO, Nzimande SB T, Mkize NG. 2015. Antimicrobial resistance and virulence signatures of *Listeria* and *Aeromonas* species recovered from treated wastewater effluent and receiving surface water in Durban, South Africa. *BMC Microbiol* 15: 234. DOI: 10.1186/s12866-015-0570-x.
- Parker JL, Shaw JG. 2011. *Aeromonas* spp. clinical microbiology and disease. *J Infect* 62 (2): 109-118. DOI: 10.1016/j.jinf.2010.12.003.
- Percival SL, Williams DW. 2014. *Aeromonas*. In: Percival SL, Williams DW, Gray FN, Yates MV, Chalmers RM. (eds). *Microbiology of waterborne diseases: Microbiological aspects and risks*. 2nd edition. Elsevier, London. DOI: 10.1016/B978-0-12-415846-7.00003-2
- Sellegounder D, Gupta YR, Muruganankumar R, Senthilkumaran B. 2018. Enterotoxic effects of *Aeromonas hydrophila* infection in the catfish, *Clarias gariepinus*: Biochemical, histological and proteome analyses. *Vet Immunol Immunopathol* 204: 1-10. DOI: 10.1016/j.vetimm.2018.08.008.
- Shakil S, Khan R, Zarrilli R, Khan AU. 2008. Aminoglycosides versus bacteria-a description of the action, resistance mechanism, and nosocomial battleground. *J Biomed Sci* 15 (1): 5-14.
- Simon SS, Lalitha KV, Joseph TC. 2016. Virulence properties of *Aeromonas* spp. from modified-atmosphere- and vacuum-packed milk fish (*Chanos chanos* Forsskal, 1775). *Ann Microbiol* 66 (3): 1109-1115. DOI: 10.1007/s13213-016-1193-7.
- Soares GMS, Figueiredo LC, Faveri M, Cortelli SC, Duarte PM, Feres M. 2012. Mechanisms of action of systemic antibiotics used in periodontal treatment and mechanisms of bacterial resistance to these drugs. *J Appl Oral Sci* 20 (3): 295-309. DOI: 10.1590/s1678-77572012000300002
- Stoll C, Sidhu JPS, Tiehm A, Toze S. 2012. Prevalence of clinically relevant antibiotic resistance genes in surface water samples collected from Germany and Australia. *Environ Sci Technol* 46 (17): 9716-9726. DOI: 10.1021/es302020s

- Stratev D, Vashin I, Daskalov H. 2013. Antimicrobial resistance of  $\beta$ -haemolytic *Aeromonas hydrophila* isolated from rainbow trouts (*Oncorhynchus mykiss*). *Boll J Vet Med* 16 (4): 289-296.
- Stratev D, Odeyemi OA. 2016. Antimicrobial resistance of *Aeromonas hydrophila* isolated from different food sources: A mini-review. *J Infect Public Health* 9 (5): 535-544. DOI: 10.1016/j.jiph.2015.10.006.
- Sumino SA, Wardiyanto. 2013. The effectiveness of ketapang (*Terminalia cattapa* L.) leave extract for the treatment of *Aeromonas salmonicida* infection in catfish (*Pangasioniodon hypophthalmus*). *Jurnal Sain Veteriner* 31 (1): 79-88. [Indonesian].
- Tipmongkolsilp N, del Castillo CS, Hikima J, Jung T, Kondo H, Hirono I, Aoki T. 2012. Multiple drug-resistant strains of *Aeromonas hydrophila* isolated from tilapia farms in Thailand. *Fish Pathol* 47 (2): 56-63.
- Tjay TH, Rahardja K. 2015. Important Medicines: Benefits, Uses and Side Effects. 7th edition. Elex Media Komputindo, Jakarta. [Indonesian].
- Varela AR, Nunes OC, Manaia CM. 2016. Quinolone resistant *Aeromonas* spp. as carriers and potential tracers of acquired antibiotic resistance in hospital and municipal wastewater. *Sci Total Environ* 542: 665-671. DOI: 10.1016/j.scitotenv.2015.10.124.
- Vega-Sánchez V, Acosta-Dibarrat J, Vega-Castillo F, Castro-Escarpulli G, Aguilera-Arreola MG, Soriano-Vargas E. 2014. Phenotypical characteristics, genetic identification, and antimicrobial sensitivity of *Aeromonas* species isolated from farmed rainbow trout (*Oncorhynchus mykiss*) in Mexico. *Acta Trop* 130: 76-79. DOI: 10.1016/j.actatropica.2013.10.021
- Verner-Jeffreys DW, Welch TJ, Schwarz T, Pond MJ, Woodward MJ, Haig SJ, Rimmer GSE, Roberts E, Morrison V, Baker-Austin C. 2009. High prevalence of multidrug-tolerant bacteria and associated antimicrobial resistance genes isolated from ornamental fish and their carriage water. *PLoS One* 4 (12): e8388. DOI: 10.1371/journal.pone.0008388.
- Wang L, Wei Y, Yuan G, Dai M, Chen X. 2012. Molecular characterization and virulence genes of *Aeromonas hydrophila* isolated from the Chinese giant salamander (*Andrias davidianus*). *Asian Herpetol Res* 3 (4): 303-309. DOI: 10.3724/SP.J.1245.2012.00303.
- Wimalasena SHMP, de Silva BCJ, Hossain S, Pathirana HNKS, Heo GJ. 2017. Prevalence and characterisation of quinolone resistance genes in *Aeromonas* spp. isolated from pet turtles in South Korea. *J Glob Antimicrob Resist* 11: 34-38. DOI: 10.1016/j.jgar.2017.06.001.
- Yano Y, Hamano K, Tsutsui I, Aue-Umneoy D, Ban M, Satomi M. 2015. Occurrence, molecular characterization, and antimicrobial susceptibility of *Aeromonas* spp. in marine species of shrimps cultured at inland low salinity ponds. *Food Microbiol* 47: 21-27. DOI: 10.1016/j.fm.2014.11.003.
- Zhou Y, Yu L, Nan Z, Zhang P, Kan B, Yan D, Su J. 2019. Taxonomy, virulence genes and antimicrobial resistance of *Aeromonas* isolated from extra-intestinal and intestinal infections. *BMC Infect Dis* 19: 158. DOI: 10.1186/s12879-019-3766-0.