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Antibiotic resistance profiles of sentinel bacteria isolated from aquaculture in Cambodia

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ABSTRACT

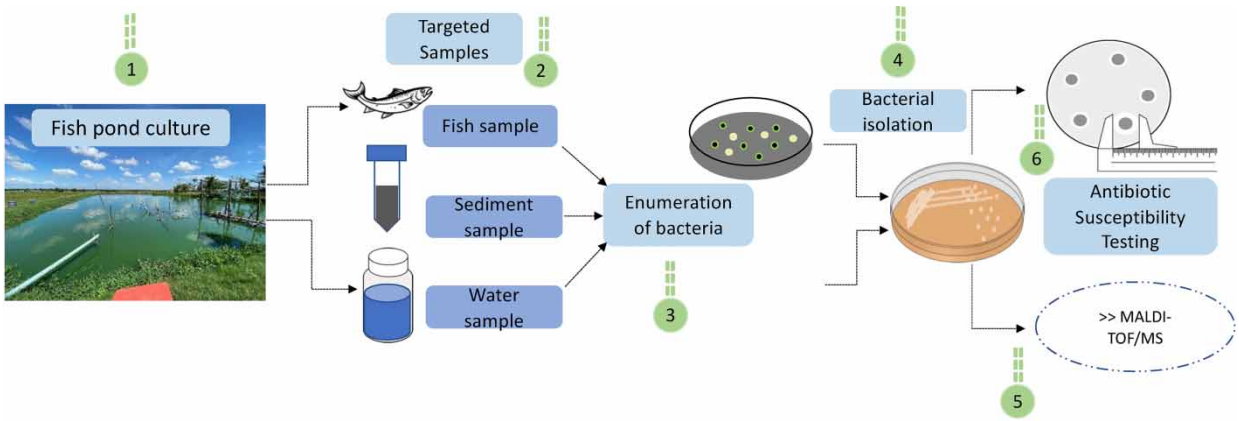
The misuse of antibiotics and the emergence of antimicrobial resistance (AMR) is a concern in the aquaculture industry because it contributes to global health risks and impacts the environment. This study analyzed the AMR of sentinel bacteria associated with striped catfish (*Pangasisanodon hypophthalmus*) and giant snakehead (*Channa micropeltes*), the two main fish species reared in the pond culture in Cambodia. Phenotypic and genotypic characterization of the recovered isolates from fish, water, and sediment samples revealed the presence of bacteria, such as 22 species belonging to families *Aeromonadaceae*, *Enterobacteriaceae*, and *Pseudomonadaceae*. Among 48 isolates, *Aeromonas caviae* (n = 2), *Aeromonas hydrophila* (n = 2), *Aeromonas ichthiosmia* (n = 1), *Aeromonas salmonicida* (n = 4) were detected. *A. salmonicida* and *A. hydrophilla* are known as fish pathogens that occur worldwide in both fresh and marine water aquaculture. Antibiotic susceptibility testing revealed antibiotic resistance patterns of 24 (50 %) isolates among 48 isolates with higher multiple antibiotic resistance index (> 0.2). All the isolates of *Enterobacteriaceae* were susceptible to ciprofloxacin. Ciprofloxacin is a frontline antibiotic that is not recommended to use in aquaculture. Therefore, its use has to be strictly controlled. This study expands our knowledge of the AMR status in aquaculture farms which is very limited in Cambodia.

Key words: antibiotic-resistant, aquaculture, Cambodia, giant snakehead, multiple antibiotic resistance index, striped catfish

HIGHLIGHTS

- Antibiotic resistance was found in enteric bacteria and *Aeromonas* spp. isolated from some Cambodian fish farms.
- Some isolates were resistant to more than one antibiotic.
- More extensive studies are required to ascertain the risks of antimicrobial resistance to Cambodian aquaculture and consumers.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Since the discovery of penicillin by A. Fleming in 1929, antibiotics from various classes have been manufactured and used globally to combat diseases caused by harmful bacteria in humans, animals, and plants. Consequently, significant amounts of antibiotics have been released into the environment through the release of wastes from households, hospitals, pharmaceutical companies, wastewater treatment plants, as well as aquaculture and livestock farms (Anh *et al.* 2021).

The consumption of fish and seafood is projected to rise by 27% by 2030, largely driven by the growth of the aquaculture industry, which is anticipated to expand by 62% in the same timeframe. Aquaculture is a crucial source of income for numerous households, supporting over 100 million individuals worldwide. Consequently, aquaculture plays a vital role in enhancing food security and reducing poverty (Reverter *et al.* 2020).

With the growing population in Cambodia, aquaculture is becoming economically important for ensuring food security. Fish is the most important source of animal protein for Cambodians, providing around 75% of the total animal protein intake for the population (Lang 2015). There are various and diverse production systems in the country, from floating cage culture, earthen pond culture, rice-fish culture, and other fish culture activities in small water bodies or aquaculture-based fisheries (FAO 2019). Aquaculture of *Pangasianodon hypophthalmus* or *Trey pra* was introduced to Cambodia and has since become a key species in the country's aquaculture due to its rapid growth, year-round production, high productivity, and high tolerance to unfavorable environmental conditions such as low dissolved oxygen, pH, and turbidity fluctuations (FiA 2019). On the other hand, giant snakeheads or *Channa micropeltes* or *Trey chhdor* in Khmer caught from the wild or imported from Vietnamese hatcheries are farmed in cages and ponds to convert low-value catches into high-value ones (Poulsen *et al.* 2008). The health of *C. micropeltes* is also a major concern, due to disease outbreaks caused by bacterial infections, especially in large-scale production, which can result in serious economic losses (Mohamad *et al.* 2020).

Antibiotics, antifungals, and other pharmaceutical drugs are administered to fish for disease prevention and treatment in aquaculture (Reverter *et al.* 2020) contributing to the global spread of antimicrobial resistance (AMR) (Done *et al.* 2015). Contamination from human and animal wastes in water effluents and, therefore, in the aquaculture environment also contributes to the increased risk (Hossain *et al.* 2022). This raises concerns about food safety and human health associated with aquaculture products. Irrational antimicrobial use, together with a lack of awareness, weak infection prevention and control, unregulated access, self-medication, inadequate training, and low-quality or counterfeit drugs, are some problems experienced in Cambodia (Om *et al.* 2016, 2017). There is no study on Knowledge Attitude and Practices (KAP) concerning the use of antibiotics for Cambodian aquaculture, but antibiotics are widely used in neighboring countries, particularly in Vietnam (Dang *et al.* 2021). A recent review found that 67 antibiotic compounds were utilized in 11 out of 15 countries between 2008 and 2018, with oxytetracycline (OT), sulfadiazine, and florfenicol (FFC) being used by 73% of these countries. On average, countries used 15 antibiotics, with top users including Vietnam, China, and Bangladesh (Lulijwa *et al.* 2020). Moreover, in Bangladesh, OT, ciprofloxacin (CIP), enrofloxacin, erythromycin (E), sulfadiazine, and trimethoprim (W) are being extensively used by fish farmers (Kawsar *et al.* 2022).

Several studies have shown that antibiotics accumulate in the culture environment, sediments, and buildup in farmed animal tissues, with consequences impacting human and environmental health (Lulijwa *et al.* 2020). For instance, the antibiotics accumulated in sediment could drive change in microbial communities through selection for antibiotic-resistant species to promote active medical antibiotics and antibiotic-resistant pathogens and genes due to increased selective pressure, rendering the drug increasingly useless (Lulijwa *et al.* 2020).

Emerging antibiotic-resistant bacteria (ARB) in aquaculture have been reported such as antibiotic-resistant *Escherichia coli*, *Acinetobacter* spp., *Aeromonas* spp., *Streptococci* and *Enterococci*, *Salmonella* spp., *Edwardsiella* spp, and *Streptococcus* spp. (Lulijwa *et al.* 2020). Due to the inherent connections between aquacultural systems with open water bodies, such as rivers and lakes, ARB have been reported to have side effects in open water systems (Lulijwa *et al.* 2020). At worst, dangerous pathogens eventually acquire resistance to all previously effective antibiotics, resulting in uncontrolled epidemics and epizootics that can no longer be treated (Lulijwa *et al.* 2020).

Knowledge of antimicrobial use in Cambodia is still limited and there are few published AMR datasets in this country. There is a clear research gap, and data on antibiotic-resistant profiles of bacteria isolated from aquaculture farms are lacking in Cambodia.

Sentinel bacteria are a group of bacteria that are monitored for their potential to cause infections and their resistance to antibiotics. Considering that *Aeromonas* spp. are useful bacterial indicators of water quality in aquaculture and a potential

indicator of antimicrobial susceptibility for the aquatic environment (Naviner *et al.* 2011), we attempt to investigate these organisms in fish and their culture environment. The incidence of AMR in fish pathogens and the aquaculture environment needs to be monitored at regular intervals to undertake effective measures for the timely prevention of bacterial diseases. In this context, the study aimed to evaluate the level of AMR of *Aeromonas* spp. and other bacteria associated with aquaculture ponds from giant snakehead and *Pangasius* fish farms.

This study aimed at providing insights into the status of AMR in Cambodia's aquaculture, contributing to a dataset for controlling and reducing AMR emergence. This study is also a baseline study for enlightening future research.

2. MATERIALS AND METHODS

2.1. Sampling location

In this study, mono- and polyculture ponds of striped catfish (*P. hypophthalmus*) and giant snakehead were randomly selected from the Kampong Trolach (KT) district in the Kampong Chhnang Province and the Kien Svay (KS) district in the Kandal Province. The study area is recognized for its vibrant aquaculture production, supplying the capital city. These aquaculture facilities are strategically located near urban centers with well-established communication and market networks. During our sample collection, we encountered some farms that had already been harvested, resulting in limited ponds available for sampling. Nonetheless, we managed to collect samples from various ponds and farms for our study. The study sites are shown in Figure 1, wherein KT is located at 104°48′00.83″E longitude and 11°55′34.4″N latitude, while the study ponds in KS are located at longitude 105°14′11.15″E and latitude 11°19′28.03″N. Fifty-five samples were collected, including fish ($n = 21$), water ($n = 17$), and sediment ($n = 17$). All samples were collected from 17 ponds of eight farms located in the two provinces. Samples were collected from four culture systems of the 17 ponds, namely, mono striped catfish (Mpa) ($n = 5$), poly striped catfish (Ppa) ($n = 2$), mono giant snakehead (Mgi) ($n = 7$), and poly giant snakehead (Pgi) ($n = 3$) (Table 1).

2.2. Sample collection

Three types of samples, including fish, water, and sediment, were collected in this study. Two fish species, striped catfish and giant snakehead, were collected from the ponds in monoculture and polyculture systems. Water samples were collected from

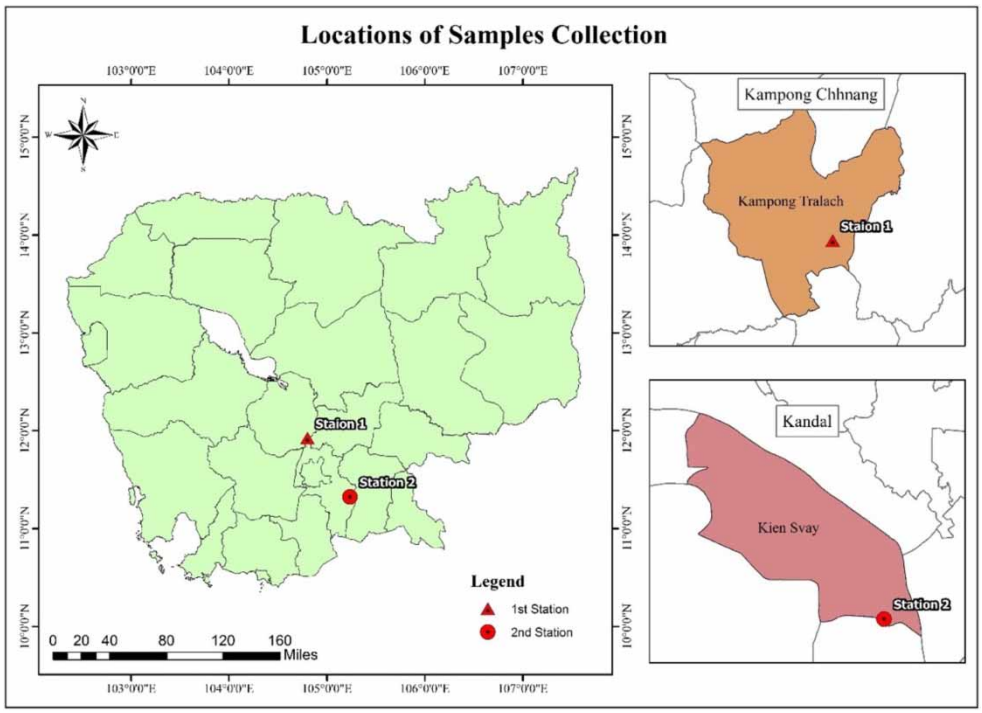


Figure 1 | Sampling locations. Station 1 is located in Kampong Trolach, Kampong Chhnang province. Station 2 is located in Kien Svay, Kandal province.

Table 1 | Number of samples collected

Province	Culture system	Pond No.	Sample number		
			Fish	Water	Sediment
KT	Mpa	4	6	4	4
	Ppa	2	0	2	2
	Mgi	2	4	2	2
	Pgi	3	6	3	3
KS	Mpa	1	1	1	1
	Mgi	5	4	5	5
Total		17	21	17	17

Mpa: Monoculture of striped catfish; Ppa: Polyculture of striped catfish; Mgi: Monoculture of giant snakehead; Pgi: Polyculture of giant snakehead.

the same cultures (ponds) as the fish samples. One water sample from each culture system was analyzed. A list of fish farmers in different locations was provided by respective fisheries officers. The first sampling was conducted on 14 June 2021 in KT, and the second sampling was conducted on 26 August 2021 in KS.

Fish were randomly collected at the sampling locations and placed into a sterile bag and killed by immersion in fusing ice, then placed in expanded boxes, and transported to the laboratory under refrigeration. They were processed within 4–6 h after collection (Scarano *et al.* 2018).

From approximately 15 cm below the water surface, 250 mL water samples were collected and poured into a sterile plastic bottle. The water samples were stored in an ice box and immediately transported to the laboratory (4–6 h).

From the same locations, approximately 100 g of sediment was collected from the pond bottom using a sediment grab (Cai *et al.* 2019). The collected sediment was placed in a sterile falcon tube and then put into an ice box during transportation to the laboratory (4–6 h). Samples were immediately processed after arrival at the laboratory.

2.3. Bacteriological cultivation and isolation

2.3.1. Fish samples

Skin, gills, and intestinal content samples were aseptically collected, weighed (3 g), and diluted with 27 mL of phosphate buffered saline (PBS) (a combination of NaCl: 8 g/L, KCl: 0.2 g/L, Na_2HPO_4: 1.44 g/L, and KH_2PO_4: 0.24 g/L) in a 1:10 (w/v) ratio. Serial dilutions up to 10^{-5} were performed. Of the homogenized sample, 0.1 mL were spread on Chromocult Coliform ES agar plates (Merck, Germany) incubated at 37 °C for 24 h and *Aeromonas* Medium Base (Ryan's medium) supplemented with ampicillin (AMP) at 5 mg/L (Oxoid, United Kingdom) and incubated at 30 °C for 48 h.

2.3.2. Water samples

Water samples were diluted into PBS in a 1:10 (v/v) ratio and vortexed for 5 s before serial dilution up to 10^{-3} in the same buffer. Volumes (0.1 ml) of each dilution were spread and treated as reported above for fish samples.

2.3.3. Sediment samples

Sediment samples were suspended in a 1:10 (w/v) ratio, vortexed for 5 min, and serially diluted up to 10^{-3} in PBS (Cai *et al.* 2019). The treatment of these samples was the same as that described for fish and water samples.

2.3.4. Isolation of presumptive *Aeromonas*

The *Aeromonas* Medium Base was used to detect the presence of *Aeromonas* species. The opaque dark green presumptive colonies with darker centers were selected for further characterization. All isolates were stored in tubes containing Luria-Bertani (LB) (Trypton: 10 g/L, yeast extract: 5 g/L, and NaCl: 5 g/L) broth with 30% v/v glycerol at −80 °C for further analysis.

2.3.5. Antibiotic susceptibility test

Each bacterial isolate was tested for antibiotic susceptibility. Antibiotic susceptibility was determined by the disk diffusion method (Baron *et al.* 2017). Inoculum was prepared by mixing three or more culture colonies in 4 mL of PBS and adjusted to the turbidity equivalent to a 0.5 McFarland standard (Remel™, United States).

The culture suspension was spread onto Mueller-Hinton agar (MHA) (HiMedia, India) (4 mm depth equivalent to 25–30 mL) in three directions using sterile swabs (~3 rotations of 60° angle). Plates were allowed to dry for a few minutes and antibiotic disks were placed at 3 cm intervals on the agar surface using a disk dispenser (Oxoid, United Kingdom). Plates were then incubated at 35 ± 2 °C for 16–18 h, and the diameter of inhibition zones was measured.

Table 2 shows the nine antibiotics and their sensitivity concentrations. The zone diameter breakpoints in this study followed the *Enterobacteriaceae* breakpoints (CLSI 2018) for *Enterobacteriaceae* and *Pseudomonadaceae* species tested against seven antibiotics such as AMP (10 μg), OT (30 μg), cefpodoxime (CPD) (10 μg), CIP (5 μg), W (1.25 μg), gentamicin (CN) (10 μg), and FFC (30 μg). *Aeromonas* spp. tested against six antibiotics such as OT (30 μg), oxolinic acid (OA) (2 μg), W (1.25 μg), E (15 μg), CN (10 μg), and FFC (30 μg), and Interpretive Categories and Zone Diameter ECVs followed *Aeromonas salmonicida* as described in VET04 (CLSI 2020). As FFC belongs to the same class as chloramphenicol and has similar characteristics, the zone diameter breakpoint of FFC follows that of chloramphenicol for *Enterobacteriaceae* and *Pseudomonadaceae* species. *E. coli* ATCC 25922 was used as a quality control strain for antibiotic disc as recommended by CLSI (2018). The multiple antibiotic resistance (MAR) index was calculated as the ratio between the number of antibiotics that an isolate is resistant to and the total number of antibiotics the organism is exposed to. A MAR greater than 0.2 means that the high-risk source of contamination is where antibiotics are frequently used.

2.4. MALDI-TOF analysis

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) is used to quickly identify microorganisms by acquiring a spectrum that is characteristic of each bacterial species (Croxatto *et al*. 2012). First, each bacterial isolate was grown on MHA agar (Oxoid, UK) for 24 h at 30 °C. Then, a sterilized loop was used to pick up a small portion of the colonies, and this inoculum was directly smeared as a thin film directly onto a sample position on the MALDI target plate. Next, 1 μL of 70% formic acid was overlaid and dried at room temperature. Then, 1 μL of HCCA matrix solution was overlaid for 30 min at

Table 2 | Antibiotics used in the study and zone diameter breakpoints

***Enterobacteriaceae* family**

			Zone diameter breakpoints (mm)			
Antibiotic class	**Antibiotics abbreviation**	**Disk concentration (μg)**	**Sensitive**	**Intermediate**	**Resistant**	**Reference**
Beta-lactam	AMP	10	≥17	14–16	≤13	CLSI (2018)
Tetracycline	OT	30	≥15	12–14	≤11	
Cephalosporin	CPD	10	≥21	18–20	≤17	
Fluoroquinolone	CIP	5	≥21	16–20	≤15	
Sulfonamide	W	1.25	≥16	11–15	≤10	
Aminoglycoside	CN	10	≥ 15	13–14	≤12	
Phenicols	FFC	30	≥18	13–17	≤12	

***Aeromonadaceae* family**

			Interpretive categories and zone diameter ECVs, nearest whole (mm)		
Antibiotic class	**Antibiotics abbreviation**	**Disk concentration (μg)**	**WT**	**NWT**	**Reference**
Tetracycline	OT	30	≥28	≤27	
Fluoroquinolone	OA	2	≥30	≤29	
Cephalosporin	CPD	10	≥21	≤20	
Sulfonamide	W	1.25	≥14	≤13	
Aminoglycoside	CN	10	≥18	≤17	
Phenicols	FFC	30	≥27	≤26	CLSI (2020)

WT, wild-type strain which is antibiotic susceptible; NWT, non-wild-type strain which is antibiotic-resistant.

room temperature. The target was ready for analysis using the MALDI-TOF bench (Bruker Daltonics, Microflex) (Kanak & Yilmaz 2019).

3. RESULTS AND DISCUSSION

3.1. Isolation, identification, and qualitative composition of bacterial isolates

Forty-eight (17 from fish samples, 23 from water samples, and 8 from sediment samples) were screened from all 17 ponds of the study sites. Those appearing dark green, opaque, and with the dark center were confirmed to be *Aeromonas* spp. by MALDI-TOF. The results from MALDI-TOF confirmed that only nine isolates identified as *Aeromonas spp*., accounting for 19% of the 48 strains. Four *Aeromonas* spp. were identified, including *Aeromonas caviae* ($n = 2$), *Aeromonas hydrophila* ($n = 2$), *Aeromonas ichthiosmia* ($n = 1$), and *A. salmonicida* ($n = 4$) (Table 3). In addition, 34 isolates belonging to *Enterobacteriaceae* were found, accounting for 70%, including *Citrobacter freundii* ($n = 8$), *Citrobacter braakii* ($n = 2$), *Enterobacter cloacae* ($n = 1$), *E. coli* ($n = 4$), *Klebsiella oxytoca* ($n = 1$), *Klebsiella pneumoniae* ($n = 3$), *Plesiomonas shigelloides* ($n = 1$), *Proteus hauseri* ($n = 3$), *Proteus vulgaris* ($n = 3$), *Rahnella aquatilis* ($n = 3$), and *Rahnella inusitata* ($n = 2$). In addition, only one isolate of *Providencia alcalifaciens* was detected in the water. Two species of *Serratia*, *Serratia marcescens* ($n = 1$) and *Serratia nematodiphila* ($n = 1$), were also detected.

Four species of *Pseudomonas,* accounting for 10% of overall isolates, were identified, namely, *Pseudomonas fragi* ($n = 1$), *Pseudomonas guariconensis* ($n = 2$), *Pseudomonas putida* ($n = 1$), and *Pseudomonas stutzeri* ($n = 1$).

According to the manufacturer of *Aeromonas* medium supplemented with AMP (5 mg/L), presumptive *Aeromonas* will appear to be dark green and opaque. *A. hydrophila* must be dark green and have a dark center. Adding AMP is supposed to inhibit other non-resistant bacteria including *Enterobacteriaceae* but not *Aeromonas* spp. which are intrinsically resistant to AMP. However, results showed that 70% of species isolated in *Aeromonas* media were *Enterobacteriaceae* species, in numbers higher than those of *Aeromonas* spp. ($n = 9$). The addition of 5 mg/L AMP is not sufficient to inhibit the isolated *Enterobacteriaceae* when they are developing resistance to AMP.

Table 3 | Total numbers of bacteria isolates and their diversity

Family	Genus	Species	Number of isolates	Isolates as a percentage (%)
Aeromonadaceae	*Aeromonas*	*Aeromonas caviae*	2	19
		Aeromonas hydrophila	2	
		Aeromonas ichthiosmia	1	
		Aeromonas salmonicida	4	
Enterobacteriaceae	*Citrobacter*	*Citrobacter freundii*	8	65
		Citrobacter braakii	2	
	Enterobacter	*Enterobacter cloacae*	1	
	Escherichia	*Escherichia coli*	4	
	Klebsiella	*Klebsiella oxytoca*	1	
		Klebsiella pneumoniae	3	
	Plesiomonas	*Plesiomonas shigelloides*	1	
	Proteus	*Proteus hauseri*	3	
		Proteus vulgaris	3	
	Rahnella	*Rahnella aquatilis*	3	
		Rahnella inusitata	2	
	Providencia	*Providencia alcalifaciens*	1	2
	Serratia	*Serratia marcescens*	1	4
		Serratia nematodiphila	1	
Pseudomonadaceae	*Pseudomonas*	*Pseudomonas fragi*	1	10
		Pseudomonas guariconensis	2	
		Pseudomonas putida	1	
		Pseudomonas stutzeri	1	
Total			48	100

Total isolates were 28 from Mgi and 9 from Pgi, followed by 8 from Mpa and 3 from Ppa. Overall, 37 of the total 48 isolates were obtained from giant snakehead environment, where *Enterobacteriaceae* predominated. However, more investigation is needed to confirm this assumption.

Aeromonas spp. was detected in the three matrices (fish, water, and sediment). A recent study also found that a high frequency of *Aeromonas* spp. is responsible for fish diseases, with the interesting thing being that some strains have been associated with human disease (Huddleston *et al.* 2013). Almost 95% of human isolates are in decreasing order belonging to the species *A. caviae*, *Aeromonas veronii*, *Aeromonas dhakensis*, and *A. hydrophila* (Figueras & Beaz-Hidalgo 2015). *Enterobacteriaceae* are Gram-negative, catalase-positive, function with facultative aerobics, and are non-glucose fermenters (Kaper *et al.* 2004). These bacteria are usually associated with the gastrointestinal tract of fish. Some studies have shown that some of their species, such as *E. coli*, *Enterobacter* spp., and *K. pneumonia*, are isolated from fish in pisciculture (Yagoub 2009). Although in most cases *Enterobacteriaceae* are part of normal microbiota from fish when colonizing human organs and tissues, they can cause some diseases, like urinary tract infections (Guzmán *et al.* 2004; Nagamatsu *et al.* 2015). In other studies, *Enterobacteriaceae* such as *Edwardsiella tarda*, *S. marcescens*, *Klebsiella aerogenes*, *Proteus penneri*, *P. hauseri*, *E. cloacae*, *Enterbacter cancerogenus*, *Enterbacter ludwigii*, *C. freundii*, *E. coli*, *Kluyvera cryocrescens*, *P. shigelloides,* and *Providencia vermicola* were recovered from infected freshwater goldfish (Preena *et al.* 2021).

Aeromonas spp. are frequently isolated from diseased fish and cause infection in humans in rare cases (Huddleston *et al.* 2013). *Aeromonas* species have been found in diseased fish, including those with conditions like motile *Aeromonas* septicemia, hemorrhagic septicemia, ulcer disease, and red-sore disease (Patil *et al.* 2016). *Aeromonas* outbreaks commonly occur in aquaculture facilities, as these bacteria are highly ubiquitous in freshwater bodies. *A. salmonicida* and *A. hydrophila* are known to cause ulcerative and hemorrhagic skin ulcers in fish under stress, which is often associated with poor sanitation and nutritional deficiencies (Igbinosa *et al.* 2012; Preena *et al.* 2020). *A. hydrophila* and other *Aeromonas* species (i.e., *A. caviae*, *A. veronii*, *Aeromonas sobria*, and *Aeromonas schubertii*) are the main causative agents of hemorrhagic septicemia in warm-water fish (Gilani *et al.* 2021). Moreover, *E. coli* of the *Enterobacteriaceae* family is an indicator microorganism; its presence in environmental samples, food, or water usually indicates recent fecal contamination or poor sanitation practices, and some are even pathogenic. The important thing to consider is that both microorganisms not only infect fish but can also cause human disease (Jamal *et al.* 2020).

3.2. Antibiotic susceptibility of isolates

Figure 2 shows the antibiotic susceptibility profiles of *Aeromonas* spp. and other isolates detected in all sources. Single antibiotic resistance and the MAR index are shown in the same figure and the supplementary file.

All *Enterobacteriaceae* isolates were 100% resistant to AMP, followed by W (19%). A few isolates were resistant to OT, CPD, CN, and FFC. Two among four isolates of *E. coli* were found to be multidrug-resistant isolates (MAR > 0.2) and were resistant to AMP, CPD, W, and FFC. Unlike in our study, Reed *et al.* (2019) found that *E. coli* isolates from human samples, pigs, and chickens were resistant to AMP, fluoroquinolone, and CN. Eight out of nine *Aeromonas* isolates were resistant to W and seven out of nine were resistant to OA. Five isolates were resistant to OT, and a few were resistant to CN, FFC, and erythromycin.

Most of *Enterobacteriaceae* isolates in this study were susceptible to CIP. CIP is a second-generation fluoroquinolone antibiotic used to treat a number of bacterial infections in humans. A high prevalence of CIP-resistant *Salmonella* spp. isolates from human bloodstream infections in Cambodia have been reported (Reed *et al.* 2019). *E. coli* and *K. pneumoniae* isolated from Thailand showed high resistance rates to third-generation cephalosporins and CIP (Reed *et al.* 2019). The majority of *Salmonella* and *Campylobacter* species sampled from chicken meat retailers in Phnom Penh markets were resistant to nalidixic acid, amoxicillin, CIP, and cephalothin (Reed *et al.* 2019).

Although there was no resistance to CIP among isolates in our study, it is necessary to strictly control the use of this antibiotic, which is not recommended to use in aquaculture (Reed *et al.* 2019). A strong resistance rate for CIP was observed in *E. coli* recovered from several hospital effluents and in neighboring aquaculture sites (Girijan *et al.* 2020). This emphasizes the strong link between aquaculture and human activities through water and highlights the need for careful selection of aquaculture sites.

In our study, *Pseudomonadaceae* were resistant to AMP (5/5 isolates), CPD, W, and FFC (3/5 isolates) and sensitive to OT, CIP, and CN.

When the MAR index score is greater than 0.2, this indicates that bacteria are multidrug-resistant, namely, they are resistant to three or more antibiotics, and they have a high-risk potential (Figure 2(c)). Fourteen out of 34 (41%) bacterial isolates

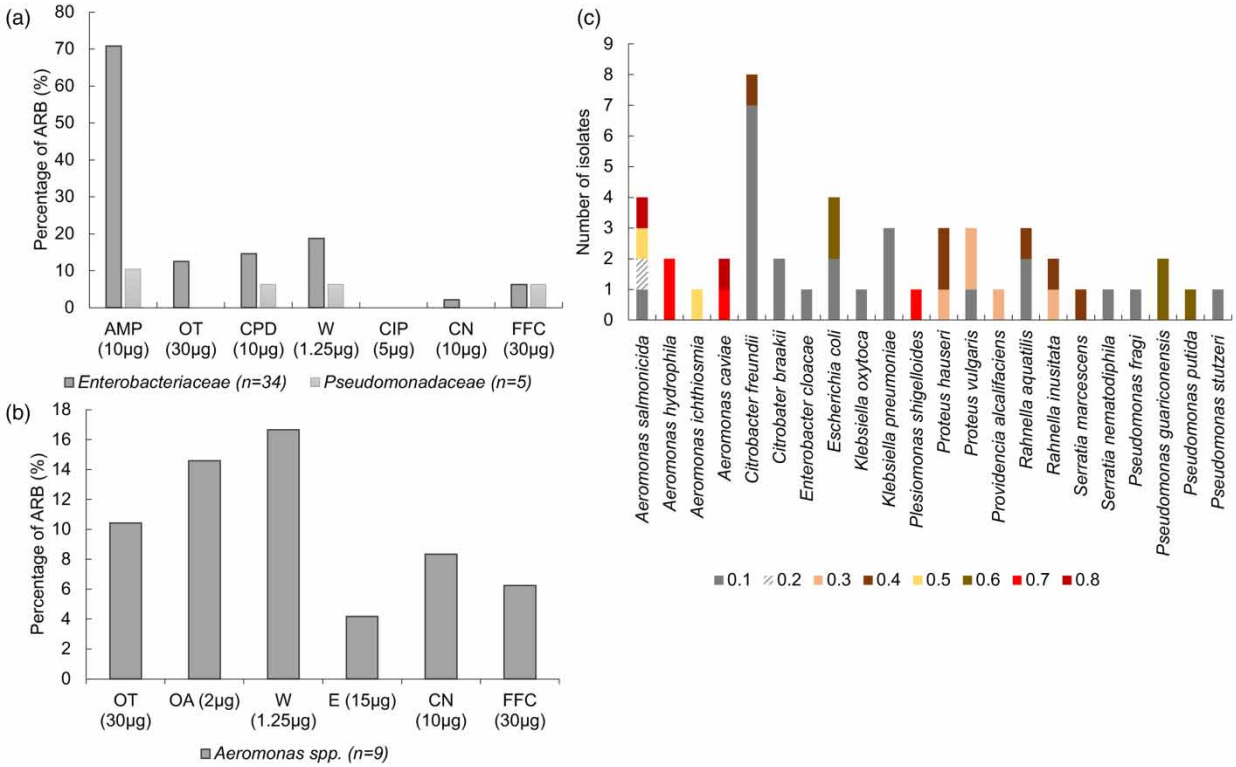


Figure 2 | (a and b) Percentage of ARB. (c) MAR index.

belonging to *Enterobacteriaceae* were found to be high-risk isolates according to the MAR index score higher than 0.2, and 2 out of 9 *Aeromonadacea*, with a score lower than 0.2. However, in most of the isolated strains (8/9 isolates), their MAR index score was higher than 0.2.

In total, 24 of the 48 isolates (50%) had an MAR index score greater than 0.2 and were multidrug-resistant. This is an alarming sign of antibiotic resistance in the aquaculture system. The presence of multiple ARB in the aquaculture environment can pose ecological risks to aquatic organisms and is responsible for the spread of antibiotic-resistant genes and ARB in aquatic ecosystems. These resistant bacteria can be transmitted to humans through food consumption and direct contact with animals and their environment. This situation raises serious concerns regarding human health and the health of the aquatic environment.

Antibiotics used in aquaculture and in human medicine are very similar (Sapkota *et al.* 2008; Naviner *et al.* 2011), and, therefore, the development of resistance of pathogens to antimicrobials important in human medicine is of utmost concern. The misuse and overuse of antibiotics have led to the emergence of ARB in the environment, an increase in antibiotic resistance in fish pathogens, the transfer of these resistance determinants to bacteria and then to terrestrial farmed animals, and finally, the development of human pathogens along with changes of the bacterial flora both in sediments and in the water. In addition, the misuse of antimicrobial agents in aquaculture can increase the prevalence of resistant bacteria that can be transmitted to humans and cause infections. Such a direct transfer of resistance from aquatic environments to humans may occur through (1) consumption of aquaculture food products or through drinking water and (2) direct contact with water or aquatic organisms or through the handling of aquaculture food products (López-bueno *et al.* 2020).

As mentioned earlier, there is no KAP for aquaculture in Cambodia, but a study conducted on duck farming (Om & McLaws 2016) reported the misuse and overuse of antibiotics at the farm level as well as a lack of awareness, weak infection prevention and control, unregulated access, self-medication, inadequate training of community health workers, and low-quality counterfeit drugs as problems rife in Cambodia. It is likely that these factors are responsible for, or contribute to, the high prevalence of AMR observed in our study.

Therefore, education on the prudent use of antibiotics in food animals and regulations are urgently needed in food animal farming in Cambodia (Om & McLaws 2016). We believe that this need is also urgent for Cambodian aquaculture.

4. CONCLUSIONS

This study highlights the widespread occurrence of AMR of sentinel bacteria associated with striped catfish (*P. hypophthalmus*) and giant snakehead (*C. micropeltes*), the two fish most reared in the pond culture environment in Cambodia. The detection of multiple ARB belonging to the families *Aeromonadaceae*, *Enterobacteriaceae*, and *Pseudomonadaceae* emphasizes the need for action to mitigate the risks associated with improper antibiotic use in aquaculture. Antibiotic susceptibility testing revealed antibiotic resistance patterns in 24 of 48 isolates, accounting for 50% with higher MAR index scores (>0.2). Overall, this study contributes to our understanding of the AMR status in aquaculture farms in the study area of Cambodia and provides valuable insights for management and reduction of antibiotic resistance in this sector.

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AUTHOR CONTRIBUTIONS

CP contributed to the conceptualization (equal), data curation (equal), formal analysis (equal), methodology (lead), validation (equal), visualization (equal), and writing the original draft (lead). SM contributed to the investigation (equal) and validation (equal). PK contributed to the investigation (equal) and validation (equal). SChe contributed to the investigation (equal) and validation (equal). CT contributed to the investigation (supporting), resources (equal), and supervision (supporting). OH contributed to data curation (equal), formal analysis (equal), and methodology (Supporting). SS contributed to the supervision (equal) and validation (equal). SCh contributed to the validation (equal). DC contributed to the conceptualization (equal), formal analysis (equal), methodology (equal), supervision (equal), validation (equal), and review and editing the manuscript writing (equal).

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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