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The Prevalence of *Vibrio cholerae* and *Vibrio parahaemolyticus* Virulence Genes and Multiple Antibiotics Resistant (MAR) Assessment from Local Shrimp Farm in Sarawak

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ABSTRACT

Excessive and improper antibiotic use in animals raised for human consumption can increase the risk of antibiotic-resistant infections, causing more harm and higher treatment costs. This study examined the virulence genes and antibiotic susceptibility of *Vibrio cholerae* and *V. parahaemolyticus*, two bacteria that can affect public health. A total of 32 water samples were collected from August to December 2021 from a shrimp farm in Sarawak. *Vibrio cholerae* (n = 10) and *V. parahaemolyticus* (n = 10) presumptive isolates were identified and purified using selective agar and duplex-PCR method. The results showed that 70% of *V. cholerae* isolates contained *rtxA* and 90% of *V. cholerae* isolates contained *rtxC* while *tdh* and *trh* were not found in *V. parahaemolyticus* isolates. Antibiotic susceptibility testing showed that all *V. cholerae* and *V. parahaemolyticus* isolates were resistant to at least one antibiotic with the mean Multiple Antibiotic Resistance (MAR) indices of 0.34 for *V. cholerae* and 0.24 for *V. parahaemolyticus*. The MAR index of 0.20 and greater indicates that antibiotics are heavily contaminating the shrimp farm water. This study highlights the need for the proper administration of antibiotics in shrimp farming environments to reduce the risk of antibiotic-resistant infections caused by *V. cholerae* and *V. parahaemolyticus*. Water treatment should also be implemented before being released back to the environment to lessen the negative impact brought by the rearing of shrimp from a highly contaminated source.

Keywords: Antibiotic resistance, shrimp farm, *Vibrio cholerae*, *V. parahaemolyticus*, virulence genes

INTRODUCTION

A significant and valuable aquaculture product, shrimps are widely traded on a global scale. Governments in developing nations like China, Thailand, Indonesia, India, Vietnam, Ecuador and Bangladesh are compelled to encourage shrimp farming as a way to fight poverty due to the high demand for shrimps (World Wildlife Fund, 2022). In Malaysia, shrimp export during 2017 and 2018 contributed RM 0.8 billion of profit in 2017 and RM 1 billion in 2018 (Fisheries Development Authority of Malaysia, 2020). According to the same source, shrimp goods also had the greatest export values when compared to other fishery products in those years, respectively. With high value of consumption of shrimp, there also comes the risk of the infection from shrimp sources. *Vibrio* species, including *V. parahaemolyticus* and *V. cholerae*, have been found to be associated with shrimp and pose a risk to human health.

Recent studies showed that *V. parahaemolyticus* have been prevalent in shrimp farms in Malaysia, Indonesia, India and Thailand (Yano *et al*., 2014; Narayanan *et al*., 2020; Sarjito & Sabdono, 2021; Haifa-Haryani *et al*., 2022). Ingesting seafood contaminated with pathogenic *V. parahaemolyticus* can cause gastroenteritis, an inflammation of the stomach and intestines that leads to symptoms such as abdominal pain, diarrhoea, vomiting and fever (Broberg *et al*., 2011). While most cases of *V. parahaemolyticus* infection are mild, in rare cases it can cause a more severe form of the disease that requires hospitalisation (Broberg *et al*., 2011). The looming

effects brought by *V. parahaemolyticus* serves as a motivation for it to be included in this study.

In addition to *V. parahaemolyticus*, the presence of *V. cholerae* in shrimp is also prominent (Joseph *et al*., 2015; Kriem *et al*., 2015; Shishehchian, 2020). The presence of *V. cholerae* in shrimp is a particularly concerning issue, as it is responsible for causing cholera infection towards human (Fu *et al*., 2019). Cholera infection causes severe dehydration with the onset of rice water diarrhoea and can cause a healthy person to die within 24 hours if not treated (Dick *et al*., 2012). Given the long track record of *V. cholerae* as a food-borne pathogen (Reda *et al*., 2022), it is not surprising that this bacterial species was also included in the current study.

Vibrio is a natural occurring microbiota of shrimp guts; therefore, they are not necessarily pathogenic (Zoqratt *et al*., 2018). However, an unhealthy shrimp harboured 30% more *Vibrio* in their guts of overall shrimp gut microbiome, compared to a healthy shrimp (Kuthoose *et al*., 2021). Their ability to cause infections can be determined by the presence of virulence genes in their genome. Thermostable direct haemolysin (*tdh*) and thermostable-related haemolysin (*trh*) are majorly related to the toxicity of *V. parahaemolyticus* (Raghunath, 2015). The expression of *tdh* causes the formation of pore in red blood cell membrane (Matsuda *et al*., 2010). *trh* genes are 70% similar to that of *tdh* and the expression results in abnormal secretion of chloride ion in human colon (Takahashi *et al*., 2000). Repeat in toxin genes (*rtxA* and *rtxC*) are recently found in abundance in the genome of *V. cholerae* (Fu *et al*., 2020). *rtxA* and *rtxC* are present in many Gram-negative bacteria and can be transferred across their envelope (Linhartová *et al*., 2010). *rtxA* and *rtxC* genes expression induces cell death in human intestine (Lee *et al*., 2008). Cholera toxin subunit B (*ctxB*) is the gene responsible for the watery diarrhoea symptom causing dehydration and severe electrolyte imbalance in human (Satitsri *et al*., 2016). *ctxB* was also detected in shrimp and is hazardous towards public health when consumed (Madhusudana & Surendran, 2013).

In shrimp farms, illnesses brought on by *Vibrio* species are typically treated with antibiotics such as tetracycline, norfloxacin, oxytetracycline, enrofloxacin and sulphonamides (Holmström *et al*., 2003; Luu *et al*., 2021). They were added in shrimp feed, or in the rearing pond water. However, due to the improper administration of antibiotics, the cases of antibiotic resistant bacteria have emerged (Holmström *et al*., 2003; Fletcher, 2015). Over the past few decades, the resistance towards antibiotics has developed and spread throughout many bacterial species as a result of the overuse of antibiotics in agricultural and aquaculture systems (Cabello, 2006). Antibiotic resistance is the term used to describe a bacteria increased in ability to withstand the effects of antibiotics, to which they were previously vulnerable to (Osunla & Okoh, 2017). Even non-pathogenic bacteria can harbour antibiotic resistance genes that then can transfer to pathogenic bacteria by a mechanism called horizontal gene transfer (Pérez-Rodríguez & Mercanoglu Taban, 2019). When infection can no longer treated by first-line antibiotic, more expensive and toxic medication will come in play (Ventola, 2015). Antibiotic resistance can cause longer treatment duration, increase healthcare costs that can become a burden for families, society and the economy (Ventola, 2015).

This research project was conducted with great rigor, involving an in-depth analysis of a single, intensively sampled population of *Vibrio* isolates present in the local shrimp farm water. The study spanned a period of eight months and aimed to shed light on the virulence and antibiotic resistant profile of these bacterial strains. Specifically, this research aimed to identify several key virulence genes, including *tdh*, *trh*, *ctxB*, *rtxA* and *rtxC*, which are associated with the pathogenicity of *Vibrio* species.

In addition to the investigation of virulence genes, the present study also focused on the emergence of Multiple Antibiotic Resistance (MAR) index. This index was used to identify the number of antibiotics that each isolate is resistant to, and their patterns of antibiotic resistance, with the aim of developing strategies to mitigate the potential for infection and ensure effective treatment in the near future. Overall, this study

provides valuable new insights into the virulence and antibiotic resistance of *Vibrio* isolates present in shrimp farm water, and underscores the importance of ongoing research efforts to identify and mitigate potential health risks associated with these bacterial strains.

MATERIALS AND METHODS

Water Sampling

Water sampling was commenced biweekly in two shrimp ponds (Pond A and Pond B), effluent and influent water of Persatuan Nelayan Kawasan Satang Biru, Telaga Air, starting from August to December 2021. The sampling campaign was executed within the shrimps' post-larvae stocking to harvesting time frame. A total of six (n = 6) samples from pond A, six (n = 6) samples from pond B, ten (n = 10) samples from effluent and ten (n = 10) samples from influent were successfully collected, making up to (n = 32) number of samples excluding triplicates samples. The samples were then transported to the laboratory under aseptic condition within 2 h after sampling.

Enrichment of *Vibrio* Species in Water Sample

A total of 1 ml of the water sample was pipetted into 9 ml of Alkaline Peptone Water (APW) (HiMedia, India) to enrich *Vibrio* species in the samples. This step was repeated for all samples collected. The cultures were then incubated overnight at 37 °C.

Isolation of *Vibrio* Species Using Thiosulfate-Citrate-Bile Salts Sucrose (TCBS) Agar

A loopful of broth were streaked on the surface of TCBS agar (HiMedia, India) and subsequently incubated overnight at 37 °C. Ten yellow and 10 green colonies that were formed on the agar was reintroduced to 5 ml of APW and incubated overnight at 37 °C for the detection using PCR.

Vibrio Species DNA Extraction Using Boiling Cell Method

The method was adjusted from Peng *et al*. (2013) to fit the requirement of this study. A total of 500 µl of the culture broth were pipetted into 1.5 ml microcentrifuge tube and centrifuged at 10,000 rpm for 5 min. The supernatant was discarded before adding 100 µl of deionised distilled water (ddH_2O). The mixture was then boiled for 10 min, and snap cooled in ice for 5 min. Lastly, each tube was centrifuged for 10 min at 10,000 rpm. The clear solution is the product of the DNA extract.

Polymerase Chain Reaction (PCR) for the Detection of *Vibrio parahaemolyticus* and *Vibrio cholera* Toxin Genes

PCR was done by adding exTEN PCR 2X Master Mix (Base Asia), two pairs of forward and reverse primers as specified in Table 1, ddH_2O and DNA extracts from each sampling. The volume of each reagent is shown in Table 2. Amplification of DNA sequence was done by using thermal cycler (Eppendorf, Germany) with the conditions shown in Table 3. *trh* and *tdh* (Bio basic, Canada) PCR condition was derived from Hossain *et al*. (2020) with slight modification. The PCR condition for *ctxB* (IDT, USA) was derived from Said *et al*. (1995) while *rtxA* and *rtxC* (IDT, USA) was derived from Chow *et al*. (2001), also with slight modifications.

Agarose Gel Electrophoresis (AGE)

The PCR products were loaded into 1.5% agarose gel and were charged with 80 V electric current for 1 h prior to being stained for 45 min with 0.1% of ethidium bromide (EtBr). The bands formed were then observed by using UV transilluminator before being photographed.

Antibiotic Susceptibility Test

Antimicrobial susceptibility testing was conducted to assess the ability of bacteria to either resist or be sensitive towards different antibiotics. The approach utilized in this study was based on the disc diffusion method (Kirby-Bauer, 1966). Firstly, cultures of *V. cholerae* and *V. parahaemolyticus* were isolated and suspended in inoculums to compare the turbidity with a 0.5 McFarland standard suspension. Next, these pure bacterial cultures were evenly spread on Mueller Hinton Agar (MHA) (HiMedia, India) plates supplemented with 2% NaCl using sterilized cotton swabs. After allowing the plates to settle

and dry for approximately 5 min (Uddin et al., 2018), antibiotic-containing discs were carefully placed on top of the agar surface using sterile tweezers. It is important to maintain appropriate spacing between each disc to ensure accurate measurement of results. Following placement, the plates went through an overnight incubation period at a constant temperature of 37 °C. The results were then recorded by measuring the diameter of inhibition zones formed around each disc in millimetres. These measurements will categorize the isolates response as Resistant (R), Intermediate (I), or Sensitive (S) with the guideline form Clinical and Laboratory Standards Institute - M45 (2015).

Table 1. Primer pairs for the detection of toxin genes

Target genus or species	Target gene	Primer name	Primer sequences	PCR base pair
V. parahaemolyticus	*tdh*	F-*tdh*	5'- GTA AAG GTC TCT GAC TTT TGG AC -3'	623
		R-*tdh*	5'- TGG AAT AGA ACC TTC ATC TTC ACC -3'	
	trh	F-*trh*	5'- TTG GCT TCG ATA TTT TCA GTA TCT-3'	460
		R-*trh*	5' – CAT AAC AAA CAT ATG CCC ATT TCG G-3'	
V. cholerae	*ctxB*	*ctx* B2	5'- GGT TGC TTC TCA TCA TGG AAC CAC – 3'	460
		ctx B3	5' – GAT ACA CAT AAT AGA ATT AAG GAT G-3'	
	rtxA	*rtx A*-F	5'- CTG AAT ATG AGT GGG TGA CTT ACG -3'	417
		rtx A-R	5'- GTG TAT TGT TCG ATA TCC GCT ACG -3'	
	rtxC	*rtx C*-F	5'- CGA CGA AGA TCA TTG ACG AC -3'	263
		rtx C-R	5'- CAT CGT CGT TAT GTG GTT GC -3'	

Table 2. PCR reagent for *tdh*, *trh ctxB, rtxA* and *rtxC*

PCR Reagent	Volume per reaction (µl)		
	tdh and *trh*	*ctxB*	*rtxA* and *rtxC*
exTEN 2X PCR Master Mix (Base Asia)		12.5	
10 pmol/µL of primer F-*tdh*	1.25		
10 pmol/µL primer R-*tdh*	1.25		
10 pmol/µL primer F-*trh*	1.25		
10 pmol/µL primer R-*trh*	1.25		
10 pmol/µL of primer *ctx* B2		1.5	
10 pmol/µL primer *ctx* B3		1.5	
10 pmol/µL of primer *rtx A*-F			1.5
10 pmol/µL primer *rtx A*-R			1.5
10 pmol/µL primer *rtx C*-F			1.5
10 pmol/µL primer *rtx C*-R			1.5
Nuclease-free water	3.0	1.5	3.5
DNA extract	2.0	3.0	3.0
Total volume	22.5	20	25

Table 3. PCR condition for *tdh*, *trh ctxB, rtxA* and *rtxC*

Step Cycle	**Temperature/Time**	
	tdh and *trh*	*ctxB, rtxA* and *rtxC*
Initial denaturation	94 °C (5 min)	95 °C (5 min)
Denaturation	94 °C (1 min)	95 °C (1 min)
Annealing	58 °C (1 min) (30 cycles)	55 °C (1 min) (30 cycles)
Extension	72 °C (1 min)	72 °C (1 min)
Final extension	72 °C (10 min)	72 °C (10 min)

The 20 types of antibiotics (Oxoid, UK) used in this study are as follows: Ampicillin (10 µg), amoxycillin/clavulanate (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cephalothin (30 µg), imipenem (10 µg), meropenem (10 µg), amikacin (30 µg), kanamycin (30 µg), neomycin (30 µg), streptomycin (10 µg), tetracycline (30 µg), nalidixic acid (30 µg), norfloxacin (10 µg), ciprofloxacin (5 µg), compound sulphonamides (300 µg), sulfamethoxazole (25 µg), sulfamethoxazole-trimethoprim (25 µg), chloramphenicol (30 µg) and rifampicin (5 µg).

By quantifying the number of resistant isolates, the information such as the most effective and/or ineffective antibiotics, the Multiple Antibiotic Resistant (MAR) indices, the recognition and frequency of antibiotic pattern were extrapolated.

Figure 1. PCR result for the detection of *ctxB* toxin gene in *V. cholerae* isolates as viewed in 1.5% agarose gel. M: 100 bp DNA ladder, 1: positive control for *ctxB*, 2: negative control, 3: Isolate 1, 4: Isolate 2, 5: Isolate 3, 6: Isolate 4, 7: Isolate 5, 8: Isolate 6, 9: Isolate 7, 10: Isolate 8, 11: Isolate 9, 12: Isolate 10. No *ctxB* gene were detected in the *V. cholerae* isolates

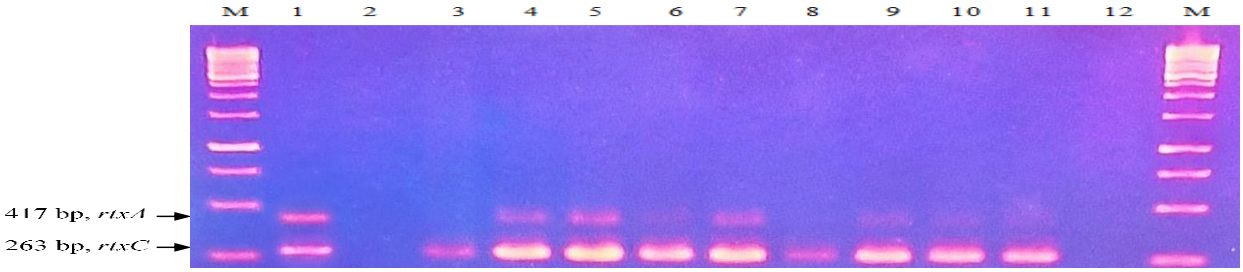

Figure 2. Duplex-PCR result for the detection of *rtxA* and *rtxC* toxin genes in *V. cholerae* isolates as viewed in 1.5% agarose gel. M: 100 bp DNA ladder, 1: positive control for *rtxA* and *rtxC*, 2: negative control, 3: Isolate 1, 4: Isolate 2, 5: Isolate 3, 6: Isolate 4, 7: Isolate 5, 8: Isolate 6, 9: Isolate 7, 10: Isolate 8, 11: Isolate 9, 12: Isolate 10. Lane 4, 5, 6, 7, 9, 10, and 11 appeared to have positive detection of *rtxA* while lane 3 to 11 showed positive detection of *rtxC* toxin gene

Figure 3. Multiplex-PCR result for the detection of *tdh*, *trh* and *tlh* toxin genes in *V. parahaemolyticus* isolates as viewed in 1.5% agarose gel. M: 100 bp DNA ladder, 1: positive control for *tdh* and *trh*, 2: negative control, 3: Isolate 1, 4: Isolate 2, 5: Isolate 3, 6: Isolate 4, 7: Isolate 5, 8: Isolate 6, 9: Isolate 7, 10: Isolate 8, 11: Isolate 9, 12: Isolate 10. Negative detection can be observed for *tdh* and *trh*

RESULTS

Toxin Genes: *Vibrio cholerae*

ctxB gene. In Figure 1, the genomes of all *V. cholerae* isolates lacked formation of any DNA bands, therefore indicating a complete absence of the said gene in the isolates.

rtxA and *rtxC* genes. Seven out of the 10 isolates showed a positive result for the detection of *rtxA*, indicating the presence of this virulence gene in those isolates. Similarly, nine out of the 10 isolates tested positive for the detection of *rtxC*, suggesting the presence of this virulence gene in those isolates. These also mean that 70% of the *V. cholerae* isolates harbour *rtxA* genes and 90% of the *V. cholerae* isolates harbour *rtxC* genes. These findings are presented in Figure 2, which clearly showed the distribution of the positive results by the formation of stained DNA bands for each virulence gene across the lanes.

Toxin Genes: *Vibrio parahaemolyticus*

tdh and *trh* genes. All 10 *V. parahaemolyticus* isolates showed no evidence of the *tdh* and *trh* gene in their genomes, as demonstrated in Figure 3. This is also determined by the lack of stained DNA bands formation indicating a complete absence of both genes in the isolates.

Antibiotic Profile: *Vibrio cholerae*

With CLSI-M45 (2015) as the reference, there appears to be no evidence of resistance from *V. cholerae* isolates, imipenem, tetracycline and norfloxacin; thus awarding them as the most potent antibiotics (Table 4). Amoxycillin/clavulanate, sulfamethoxazole-trimethoprim, and chloramphenicol are next in line and were recorded with 80% susceptibility of eradicating *V. cholerae* population. The least effective antibiotics at preventing the growth of *V. cholerae* is sulfamethoxazole (100% resistance), followed by cephalothin (80% resistance), neomycin (70% resistance), streptomycin (70% resistance) and ceftriaxone (60% resistance).

Antibiotic Profile: *Vibrio parahaemolyticus*

All of *V. parahaemolyticus* isolates were susceptible to ceftazidime, ceftriaxone, amikacin, neomycin, nalidixic acid, ciprofloxacin, sulfamethoxazole-trimethoprim and chloramphenicol (Table 5). Ninety percent of susceptibility was displayed by the antibiotic kanamycin, tetracycline and norfloxacin in eradicating *V. parahaemolyticus*. Others such as amoxycillin/clavulanate, meropenem and rifampicin were recorded to have 80% susceptibility towards *V. parahaemolyticus* isolates. The least effective antibiotics at preventing the growth of *V. parahaemolyticus* were cephalothin, streptomycin and sulfamethoxazole with 100% resistance recorded. The next antibiotic that the isolates were highly resistant to was compound sulphonamides which can be attributed to the high rates of resistance found in *V. parahaemolyticus* (80%).

MAR Indexes and Antibiotic Resistance Pattern: *Vibrio cholerae*

Table 6 shows nine patterns of antibiotic resistance with the most frequent being pattern C. The frequency happened to be of 20% occurrence of all the 10 isolates, with the MAR index of 0.25. MAR indexes of *V. cholerae* isolates range from 0.15 to 0.55 while their mean is 0.34. The antibiotic pattern I has the highest MAR index at a staggering 0.55 while the pattern A showed the lowest MAR index at 0.15. Both occurrences were recorded to be at 10% frequency. From the 10 isolates, a total of nine isolates showed MAR indexes greater than 0.2.

MAR Indexes and Antibiotic Resistance Pattern: *Vibrio parahaemolyticus*

From *V. parahaemolyticus* isolates, eight patterns of antibiotic resistance were recorded (Table 7). The most frequent pattern was B with the MAR index of 2.0. All of *V. parahaemolyticus* isolates were detected with the MAR indexes of 0.2 and higher. MAR indexes of *V. parahaemolyticus* isolates ranges from 0.20 to 0.35, making them all above 0.20 threshold. Their calculated mean is 0.24. The greatest MAR index is 0.35 from pattern

H while the lowest is pattern A with 0.20 MAR index.

DISCUSSION

In the present study, the presence of toxin genes for both *V. cholerae* and *V. parahaemolyticus* in a shrimp farm setting was determined. The presence of virulence genes, such as *tdh* and *trh* in *V. parahaemolyticus* and *rtxA* and *rtxC* in *V. cholerae*, indicates the toxicity and pathogenicity of these bacteria, and can lead to serious gastrointestinal issues when consumed. Table 8 shows the summary of PCR toxin genes detection in both *V. cholerae* and *V. parahaemolyticus* isolates that were done in this study. Aside from that, the level of susceptibility of the species towards antibiotics were also profiled to analyse the best and the worst treatment for vibriosis.

The *trh* and *tdh* genes were absent in *V. parahaemolyticus* isolates. Low detection of *tdh* and *trh* genes from aquaculture sample was also reported from previous study (Vieira *et al*., 2011). Since these virulence factors are major and can infect both people and aquatic organisms, it is crucial that their density remained low (Robert-Pillot *et al*., 2004). Despite the absence of *tdh* and *trh* genes, there is however, no certainty that *V. parahaemolyticus* does not possess other virulence components that are not covered in this study.

Table 4. Antibiotic profiling of *V. cholerae* isolates against selected antibiotics with CLSI-M45 (2015) as the reference

Antimicrobial Class	Antibiotic Names	Abbreviation	No. and Percentage of *V. cholerae* according to their Susceptible, Intermediate and Resistance Distribution		
			Susceptible, S	Intermediate, I	Resistant, R
Penicillins and β- lactam/β-Lactamase Inhibitor Combinations	Ampicillin	AMP10	4 (40%)	1 (10%)	5 (50%)
	Amoxycillin/ Clavulanate	AMC20	8 (80%)	0 (0%)	2 (20%)
Cephalosporins/Cephems	Ceftazidime	CAZ30	7 (70%)	0 (0%)	3 (30%)
	Ceftriaxone	CRO30	4 (40%)	0 (0%)	6 (60%)
	Cephalothin	KF30	1 (10%)	1 (10%)	8 (80%)
Carbapenems	Imipenem	IPM10	10 (100%)	0 (0%)	0 (0%)
	Meropenem	MEM10	7 (70%)	2 (20%)	1 (10%)
Aminoglycosides	Amikacin	AK30	4 (40%)	3 (30%)	3 (30%)
	Kanamycin	K30	3 (30%)	5 (50%)	2 (20%)
	Neomycin	N30	3 (30%)	0 (0%)	7 (70%)
	Streptomycin	S10	1 (10%)	2 (20%)	7 (70%)
Tetracyclines	Tetracycline	TE30	10 (100%)	0 (0%)	0 (0%)
Quinolones/Fluoroquinolones	Nalidixic Acid	NA30	5 (50%)	0 (0%)	5 (50%)
	Norfloxacin	NOR10	10 (100%)	0 (0%)	0 (0%)
	Ciprofloxacin	CIP5	7 (70%)	3 (30%)	0 (0%)
Folate Pathway Inhibitors	Compound Sulphonamides	S3	4 (40%)	4 (40%)	2 (20%)
	Sulfamethoxazole	RL25	0 (0%)	0 (0%)	10 (100%)
	Sulfamethoxazole-Trimethoprim	SXT25	8 (80%)	0 (0%)	2 (20%)
Phenicols	Chloramphenicol	C30	8 (80%)	2 (20%)	0 (0%)
Rifamycin	Rifampicin	RD5	6 (60%)	0 (0%)	4 (40%)

Table 5. Antibiotic profiling of *V. parahaemolyticus* isolates against selected antibiotics with CLSI-M45, (2015) as the reference

Antimicrobial Class	Antibiotic Names	Abbreviation	No. and Percentage of *V. parahaemolyticus* according to their Susceptible, Intermediate and Resistance Distribution		
			Susceptible, S	Intermediate, I	Resistance, R
Penicillins and β- lactam/β-Lactamase Inhibitor Combinations	Ampicillin	AMP10	6 (60%)	0 (0%)	4 (40%)
	Amoxycillin/ Clavulanate	AMC20	8 (80%)	0 (0%)	2 (20%)
Cephalosporins/Cephems	Ceftazidime	CAZ30	10 (100%)	0 (0%)	0 (0%)
	Ceftriaxone	CRO30	10 (100%)	0 (0%)	0 (0%)
	Cephalothin	KF30	0 (0%)	0 (0%)	10 (100%)
Carbapenems	Imipenem	IPM10	5 (50%)	4 (40%)	1 (10%)
	Meropenem	MEM10	8 (80%)	1 (10%)	1 (10%)
Aminoglycosides	Amikacin	AK30	10 (100%)	0 (0%)	0 (0%)
	Kanamycin	K30	9 (90%)	1 (10%)	0 (0%)
	Neomycin	N30	10 (100%)	0 (0%)	0 (0%)
	Streptomycin	S10	0 (0%)	0 (0%)	10 (100%)
Tetracyclines	Tetracycline	TE30	9 (90%)	0 (0%)	1 (10%)
Quinolones/ Fluoroquinolones	Nalidixic Acid	NA30	10 (100%)	0 (0%)	0 (0%)
	Norfloxacin	NOR10	9 (90%)	0 (0%)	1 (10%)
	Ciprofloxacin	CIP5	10 (100%)	0 (0%)	0 (0%)
Folate Pathway Inhibitors	Compound Sulphonamides	S3	1 (10%)	1 (10%)	8 (80%)
	Sulfamethoxazole	RL25	0 (0%)	0 (0%)	10 (100%)
	Sulfamethoxazole -Trimethoprim	SXT25	10 (100%)	0 (0%)	0 (0%)
Phenicols	Chloramphenicol	C30	10 (100%)	0 (0%)	0 (0%)
Rifamycin	Rifampicin	RD5	8 (80%)	0 (0%)	2 (20%)

Table 6. Patterns of antibiotic resistance profile and MAR index of *V. cholerae* isolates

Label	Isolate Number	[a]Antibiotic Resistance Pattern	[b]MAR Index	Percentage of Occurrence (No. of Isolates/Total of isolates)
A	10	NRlS	0.15	10%
B	3	AmpCroKfNaRl	0.25	30%
C	7	KfNRdRlS	0.25	
	8		0.25	
D	2	AmcAmpCroKfNaRl	0.30	20%
E	9	KfNRdRlSSxt	0.30	
F	1	AmcAmpCroKfMemRlSxt	0.35	10%
G	5	AkCazCroKNNaRlSS3	0.45	10%
H	6	AkAmpCazCroKfNNaRdRlS	0.50	10%
I	4	AkAmpCazCroKKfNNaRlSS3	0.55	10%

[a] Antibiotics used: Amp, Ampicillin; Amc, Amoxycillin/Clavulanate; Caz, Ceftazidime; Cro, Ceftriaxone; Kf, Cephalothin; Ipm, Imipenem; Mem, Meropenem; Ak, Amikacin; K, Kanamycin; N, Neomycin; S, Streptomycin; Te, Tetracycline; Na, Nalidixic Acid; Nor, Norfloxacin; Cip, Ciprofloxacin; S3, Compound Sulphonamides; Rl, Sulfamethoxazole; Sxt, Sulfamethoxazole-Trimethoprim; C, Chloramphenicol; Rd, Rifampicin

[b] MAR Index = The number of antibiotics that the isolate is resistant to / Total number of antibiotics used

As for *V. cholerae* isolates, 70% *rtxA* and 90% *rtxC* genes were detected. RTX toxin are well known to be associated with Gram-negative bacteria (Coote, 1992). Gram-negative bacteria-related toxins have a number of effects, including haemolysin, pore formation, and cytotoxicity (Prithvisagar *et al*., 2021). The detection of *rtxA* and *rtxC* genes were also mentioned in recent studies (Xu *et al*., 2019; Fu *et al*., 2020; Igere *et al*., 2022). Since *rtx* genes can be present in not only *Vibrio* species but also in Gram-negative bacteria, it is possible that horizontal gene transfer is widespread causing the high pathogenicity (Deng *et al*., 2019; Dell'Annunziata *et al*., 2021). In order to prevent a widespread infection from endangering the health of shrimp and people, a control measure should be carried out in response to the high proportion of *rtxA* and *rtxC* genes detected in the sampling location.

Table 7. Patterns of antibiotic resistance profile and MAR index of *V. parahaemolyticus* isolates

Label	Isolate Number	[a]Antibiotic Resistance Pattern	[b]MAR Index	Percentage of Occurrence (No. of Isolates/Total of isolates)
A	3	KfNorRlS	0.20	50%
B	4	KfRlSS3	0.20	
	5		0.20	
	8		0.20	
C	10	AmpKfRlS	0.20	
D	2	KfMemRlSS3	0.25	30%
E	6	KfRdRlSS3	0.25	
F	9	AmcAmpKfRlS	0.25	
G	7	AmpKfRdRlSS3	0.30	10%
H	1	AmcAmpIpmKfRlSTe	0.35	10%

[a] Antibiotics used: Amp, Ampicillin; Amc, Amoxycillin/Clavulanate; Caz, Ceftazidime; Cro, Ceftriaxone; Kf, Cephalothin; Ipm, Imipenem; Mem, Meropenem; Ak, Amikacin; K, Kanamycin; N, Neomycin; S, Streptomycin; Te, Tetracycline; Na, Nalidixic Acid; Nor, Norfloxacin; Cip, Ciprofloxacin; S3, Compound Sulphonamides; Rl, Sulfamethoxazole; Sxt, Sulfamethoxazole-Trimethoprim; C, Chloramphenicol; Rd, Rifampicin
[b] MAR Index = The number of antibiotics that the isolate is resistant to / Total number of antibiotics used

Table 8. The summary of the PCR detection of toxin genes in the isolates of *V. cholerae* and *V. parahaemolyticus*

Vibrio species	Toxin gene	Number of positive detections	Percentage of positive detection
V. cholerae	ctxB	0/10	0%
	rtxA	7/10	70%
	rtxC	9/10	90%
V. parahaemolyticus	tdh	0/10	0%
	trh	0/10	0%

The classes of antibiotics that are most effective for the treatment of *V. cholerae* are tetracycline (tetracycline), carbapenem (imipenem) and older quinolones (norfloxacin). As for the treatment against *V. parahaemolyticus*, cephalosporins/cephems (ceftazidime and ceftriaxone), aminoglycosides (amikacin and neomycin), older quinolones (nalidixic acid and ciprofloxacin), folate pathway inhibitor (sulfamethoxazole-trimethoprim) and phenicol (chloramphenicol) are the most effective in this study. Norfloxacin was the most effective antibiotic in eliminating all of *V. cholerae* isolates and 90% of *V. parahaemolyticus* isolates. However, despite its effectiveness, norfloxacin has been prohibited for use on food animals in Malaysia due to concerns about its potential impact on human health (Hassali *et al*., 2018). Avoparcin, chloramphenicol, nitrofurantoin, nitrofurazone, furazolidone, furaltadone, teicoplanin and vancomycin are also banned in food animals due to the level of harmful residues that can cause adverse impact on human health (Hassali *et al*., 2018). Antibiotic residues are proven to be difficult to extinguish entirely since they can remain in food even after cooking (Fathy Mahmoud, 2015).

Mean MAR indices of 0.34 for *V. cholerae* and 0.24 for *V. parahaemolyticus* were calculated in this study. Ninety percent of *V. cholerae* isolated have recorded MAR index of higher than 0.2 while 100% of *V. parahaemolyticus* have recorded MAR index of 0.2 and/or higher. MAR index greater than 0.2 indicate that isolates came from a highly contaminated source where antibiotics are

frequently used (Davis & Brown, 2016). Based on the result, *V. parahaemolyticus* displayed significant resistance towards sulfamethoxazole, compound sulphonamides, cephalothin and streptomycin. It matches the antibiotics mentioned in CLSI-M45 (2015). As for *V. cholerae*, they are the most resistant towards sulfamethoxazole, cephalothin, neomycin, streptomycin and ceftriaxone. This result is in favour with previous research that found the same antibiotics resistance from *V. cholerae* towards listed antibiotics except for neomycin (Noorlis *et al*., 2011; Mandal *et al*., 2012; Fu *et al*., 2020; Adesiyan *et al*., 2021). Low resistance and high susceptibility of neomycin for the treatment of *V. cholerae* have been recorded in previous research (Mrityunjoy *et al*., 2013; Verma *et al*., 2019). Decrease in neomycin resistant from the year 2000 to 2022 were also documented (Wu *et al*., 2023). Regardless, the use of neomycin for treating vibriosis in aquaculture and cholera infection has indeed described by other studies (Manjusha & Sarita, 2011; Farooq & Unno, 2018; Adesiyan *et al*., 2022).

The distribution of antibiotic resistance genes is linked to various factors such as potential host genera and biomes, sample collection year, environmental factors influenced by human activities and collection countries (Lin *et al*., 2022). As the shrimp farmers in this study denied any use of treatment for the shrimps, it is possible that the bacteria acquired antibiotics resistance from the environment or from the post-larvae that were imported from other hatcheries. This observation correlates with a study which stated that higher frequency of MAR occurs in hatcheries compared to in shrimp rearing pond (Zhang *et al*., 2011). Nevertheless, the high susceptibility of *Vibrio* species towards banned antibiotics in this study indicate proper obligation and understanding of shrimp farmers and aquaculture organisations towards food safety.

Several control measures can be taken before bigger conflict involving shrimp diseases develop. To avoid prolong effect of MAR, the proper usage and constant supervision of antibiotics use in shrimp farm should be implemented. The use of alternative treatments such as detergents (Elexson *et al*., 2014), probiotics (Ngo & Fotedar, 2010) and phage therapy (Chen *et al*., 2019) have all been shown to be equally effective, if not superior, in treating vibriosis in the shrimp farming industry. Water treatment should be implemented before being released back to the environment to lessen the negative impact brought by the rearing of shrimp from a highly contaminated source. These precautions should be properly considered as the shrimp farming industry develops to help safeguard the welfare of our natural environment. Finally, it is important for everyone participating in the shrimp farming sector, as well as the general public, to have a sufficient awareness when handling shellfish.

CONCLUSION

Toxin genes *rtxA* and *rtxC* were prominent in *V. cholerae* isolates, proving the pathogenicity of the bacteria. High MAR indices from both bacterial species isolates indicate the consequences of improper use of antibiotics from highly contaminated sources, whether from imported shrimp larvae from external nurseries, or other bacteria in the environment via water current. Despite the high MAR indices, the banned antibiotics are still highly susceptible towards the isolates, therefore proving the obligation of shrimp handlers towards banned antibiotics. Alternative shrimp disease treatments with lower risk towards the environment and human consumption can slowly be implemented for treating diseases caused by *Vibrio* species. Of all the antibiotics tested in this study, ciprofloxacin is the most suitable antibiotic for the treatment of vibriosis. Overall, the research presented in this study provides important insights into the pathogenicity and antibiotic resistance of *Vibrio* species isolates in aquaculture, underscoring the need for ongoing surveillance and monitoring of these bacterial strains to prevent the spread of antibiotic-resistant infections and protect the health of shrimp handlers and consumers. With the implementation of preventative measures and more sustainable practices, the impact of *Vibrio*-related illnesses on public health can be properly mitigated.

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