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Incidence, Antibiotic Susceptibility and Characterization of *Vibrio parahaemolyticus* Isolated from Seafood in Selangor, Malaysia

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Abstract: *Vibrio parahaemolyticus* is one of the major foodborne pathogens owing to its cause of infectious diseases such as gastroenteritis. These diseases are often associated with the consumption of contaminated seafood. This study aims to investigate the presence of *V. parahaemolyticus*, their virulence, antibiotic profiles, and plasmid profiles from 77 different kinds of shellfish samples collected from wet markets and supermarkets in Selangor, Malaysia. High densities of *Vibrio* species (> 5 log CFU/g) were found in 14/16 groups of shellfish. Among 77 presumptive *V. parahaemolyticus* isolates, 43 (55.8%) were positive for the *toxR* gene, confirming the identity of the isolates at the species level. However, none of the *V. parahaemolyticus* isolates harboured the virulence *tdh* and *trh* genes. The antibiotic susceptibility of the *V. parahaemolyticus* isolates revealed that most of them were resistant to ampicillin (95.3%), ampicillin-sulbactam (81.4%), cefotaxime (37.2%) and imipenem (23.3%). The plasmid profiles of the *V. parahaemolyticus* isolates showed that 41.9% (18/43) possess at least one plasmid. Our results indicate the *V. parahaemolyticus* isolates are continuously exposed to various antibiotics in the environments, thus consuming the seafood carries a potential health risk to consumers. The antibiotic resistance conferred by the species necessitates an immediate plan to approach the usage of antibiotics differently.

Keywords: *Vibrio parahaemolyticus*; shellfish; prevalence; virulence; antibiotic resistance

1. Introduction

Seafood is a well-known source of omega-3 long-chain polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid, protein, carnitine, vitamin A, B, D,

and E, and minerals (i.e., magnesium, selenium, iodine, calcium, phosphorus, iron, and zinc)[1–3]. These active compounds are shown to reduce the risk of preterm deliveries, triglyceride levels in type 2 diabetes mellitus, sarcopenia in the elderly and prostate cancer-related mortalities[3–6]. The global consumption of fish has been increasing steadily at an annual rate of 3.1% from 1961 to 2017. This is attributed to the steady increase in the production of these aquatic animals through aquaculture[7]. In 2018, the aquaculture industry produced 114.5 million tonnes of seafood consumed, in which Southeast Asia contributed to 17% of the total world production[7, 8]. The aquaculture industry of Malaysia is a significant contributor to the overall production of seafood in Southeast Asia[9]. The increasing production of aquaculture products locally is attributed to the rise in seafood demand internationally and domestically[10]. Food and Agriculture Organization (FAO) of the United Nations reported that the domestic fish intake in Malaysia is 55.9 kg per capita, thrice as much as the global average of fish consumption[9]. This finding demonstrates the importance of seafood as a source of animal protein in Malaysia. Two of the most widely exported aquaculture products in Malaysia are cockles and shrimps[9]. The microbial spoilage of seafood can occur when contaminated water, sediments and sewage runoffs enter water bodies[1,11–13]. In addition, once seafood is harvested, cross-contamination can also take place at any point during rearing, handling, preparing, processing, transporting, and storing[1,11,12]. Shellfish are prone to contamination by a plethora of organisms, resulting in shellfish-borne outbreaks[14]. Some of the most common foodborne pathogens include *Vibrio* species[15–19], *Salmonella* species[20–25], *Escherichia coli, Listeria monocytogenes*[26–30], *Plesiomonas shigelloides*, hepatitis A virus, and calicivirus[14,31].

Vibrio parahaemolyticus causes infectious diarrhoea associated with the consumption of raw or contaminated seafood[32–36]. The incubation period of the disease caused by this pathogen varies between literature. However, symptoms can occur as early as four hours post-infection[37,38]. *V. parahaemolyticus* causes gastroenteritis, which presents with watery diarrhoea, nausea, vomiting, abdominal pain, fever and chills[38]. The disease is self-limiting and lasts for approximately three days in healthy individuals[32–34]. The immunocompromised individuals may experience severe inflammatory diarrhoea, which could lead to septicaemia and death[34,39]. Pathogenic *V. parahaemolyticus* possess *tdh* and *trh* genes that encode for thermostable direct haemolysin (*tdh*) and thermostable direct haemolysin-related (*trh*), respectively, and is the primary virulence factors produced by *V. parahaemolyticus*[40,41]. These haemolysins are responsible for the enterotoxic, cytotoxic and haemolytic actions of the enteropathogen[33,34,42–46]. Environmental isolates of *V. parahaemolyticus* that do not possess *tdh* and *trh* genes produce other virulence factors to exert pathogenicity. Studies have shown that non-*tdh* and non-*trh* producing *V. parahaemolyticus* can still maintain their

enterotoxicity and cytotoxicity activities through other mechanisms, which includes but is not limited to thermolabile haemolysin (*tlh*), type III secretion systems (T3SSs) and type VI secretion system (T6SSs)[47–51].

The widespread usage of antibiotics has exerted selection pressure on bacteria, leading to resistance against these therapeutic agents. Antibiotics are excessively used in the healthcare, agriculture, and aquaculture industries[52–54]. This has resulted in the *Vibrio* species developing multi-drug resistance against common antibiotics[55–57]. Generally, bacteria may possess intrinsic resistance genes in the chromosomes or acquire resistance genes via plasmids[55,58]. Mobile genetic elements such as plasmids are transferred to other bacteria via horizontal gene transfer or vertical gene transfer. The ubiquitous existence of *Vibrio* species in aquatic animals poses a risk to humans as multi-drug resistant *Vibrio* species can be transferred directly to humans via seafood consumption[1,59]. The risk is higher in Malaysia due to the growing aquaculture industry and high consumption of seafood locally[9,10]. To date, *V. parahaemolyticus* isolated from seafood have shown resistance to β–lactams (penicillin and ampicillin), third generation cephalosporins (cefotaxime and ceftazidime), second-generation cephalosporins (cefuroxime), first-generation cephalosporins (cephalothin), bacitracin, amikacin, and vancomycin[60–62].

Frequent surveillance of the microbial status of seafood is crucial to monitor the prevalence of *V. parahaemolyticus*. The various virulence mechanisms exhibited by the pathogen contributes to the pathogenicity of bacteria, resulting in foodborne infections in human hosts. Besides that, the overuse and misuse of antibiotics have raised concerns regarding the multi-drug resistant strains of *V. parahaemolyticus*. Therefore, the focus of this study is to determine and characterise the prevalence, virulence, antibiotic resistance profile and plasmid profiles of *V. parahaemolyticus* isolated from seafood.

2. Materials and Methods

2.1 Sampling

This study included two types of bivalve mollusc (i.e., short-necked clam and blood clam) and crustacean (i.e., tiger prawn and Indian white shrimp). A total of 77 seafood samples were collected from two wet markets and two supermarkets in Selangor, Malaysia. Upon collection, the samples were kept in separate sterile sealed bags and transported to the laboratory for analysis[63].

2.2 Enumeration of Presumptive Vibrio Species

The enumeration of *Vibrio* species from seafood samples was conducted based on the Bacterial Analytical Manual of FDA and FAO/WHO Risk Assessment Tool for *Vibrio parahaemolyticus* and *Vibrio vulnificus*[63,64]. 10 g of the sample were weighed and placed in a sterile stomacher beg (Bagmixer® 400W, Interscience, France), and added with 90 mL of APW with 2% w/v NaCl, pH 8.5. The samples are stomached for 90 s. This produces the first 10^{-1} dilution[65]. The subsequent tenfold dilutions (i.e., 1:10, 1:100, 1:1000, 1:10,000) were prepared by serial dilutions[66].

HiCrome™ *Vibrio* (HiMedia™, India) agar was used in the identification of *Vibrio* species in food samples[67]. The spread plate technique was employed for the enumeration of *Vibrio* species[68]. The HiCrome™ *Vibrio* (HiMedia™, India) agar plates were inoculated with 100 µL of each dilution of the homogenate in triplicate. The agar plates were then incubated at 37°C for 18 hours. After that, the total number of *Vibrio* colonies were identified and enumerated. *V. parahaemolyticus* colonies are round, bluish-green, opaque and flat, whereas *V. cholerae* colonies are round, purple, opaque and flat on the HiCrome™ *Vibrio* (HiMedia™, India) agar plates[69].

2.3 Isolation of Presumptive Vibrio parahaemolyticus

The isolation of *Vibrio* species from seafood samples was conducted based on the FDA Bacterial Analytical Manual and FAO/WHO Risk Assessment Tool[63,64]. The homogenate in the filter bags was incubated at 37°C for 18 hours to revitalise stressed bacterial cells. After that, 50 µL of the homogenate was streaked onto the selective HiCrome™ *Vibrio* (HiMedia™, India) agar plates. The inoculated agar plates were then incubated at 37°C for 18 hours. Subsequently, presumptive *V. parahaemolyticus* colonies (based on the colony morphology) were picked and purified on Tryptic Soy Agar (TSA) (HiMedia™, India) supplemented with 2% w/v sodium chloride[70,71]. The agar plates were then incubated at 37°C for 18 hours. Vibrios generally form large, cream coloured colonies on TSA plates[66]. The purified single colonies were then kept in semisolid nutrient agar until further analysis.

2.4 Genomic DNA extraction

The genomic DNA of presumptive *V. parahaemolyticus* was extracted by the direct boiled cell lysate method, as described in previous studies[61,72]. The *V. parahaemolyticus* colonies in the semisolid nutrient agars were revived in tryptic soy broth (TSB) (HiMedia,

India) with NaCl (2% w/v) and incubated in a shaker incubator at 200 RPM and 37°C for 18 hours. One and a half millilitre of the overnight bacterial culture was transferred into microcentrifuge tubes and centrifuged for 5 minutes at 10,000 RPM. Centrifuging the bacterial suspension allows the concentration of the bacterial population in the suspension[72].

The supernatant was discarded and the pellets were resuspended in 1 mL of sterile ultrapure water, vortexed and heated at 95°C for 7 minutes in a water bath. The application of heat causes the release of bacterial DNA strands from the cells[72]. The cell lysate was then cooled in ice for 5 minutes before centrifuging for 1 minute at 10,000 RPM. Lastly, the supernatant with the nucleic acid was pipetted into new 1.5 mL microcentrifuge tubes. The DNA samples were stored at -20°C until further analysis.

2.5 Identification of Vibrio parahaemolyticus using toxR-PCR

A singleplex PCR assay targeting the *toxR* gene was employed in the molecular identification of the *V. parahaemolyticus* isolates (n = 77) at the species level (Table 6)[73]. The PCR assay was performed in a final volume of 20 µL: 2 µL of DNA template, 10 µL of 2x Taq PLUS PCR Smart Mix 1 (SolGent™, Korea), 6 µL of sterile ultrapure water, 1 µL of primer *toxR*-F and *toxR*-R each. This process was completed using the SuperCycler: ThermalCycler (Kyratec, Australia) with the following thermal conditions: pre-denaturation of DNA at 95°C for 4 minutes, 35 cycles of denaturation at 94°C for 1 minute, annealing at 68°C for 1 minute and extension at 72°C for 30 seconds and final elongation at 72°C for 5 minutes[74]. The PCR product was separated by electrophoresis in 1.5% agarose gel and visualised under a gel documentation system (ChemiDoc™ XRS, Bio-Rad, USA). *Vibrio parahaemolyticus* VP20 was employed as a positive control.

2.6 Detection of Virulence Genes, tdh and trh

The *toxR*-positive isolates were subjected to a duplex PCR assay targeting the virulence genes, *tdh* and *trh* (Table 1)[43]. The PCR assay was performed in a final volume of 20 µL: 2 µL of DNA template, 10 µL of 2x Taq PLUS PCR Smart Mix 1 (SolGent™, Korea), 4 µL of sterile ultrapure water, 1 µL of primer *tdh*-F, *tdh*-R, *trh*-F and *trh*-R each. This process was completed using the SuperCycler: ThermalCycler (Kyratec, Australia) with the following thermal conditions: pre-denaturation of DNA at 94°C for 3 minutes, 30 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, extension at 72°C for 1 minute and final elongation at 72°C for 5 minutes[74]. The PCR product was separated by electrophoresis in 1.5% agarose gel and visualised under a gel documentation system

(ChemiDoc™ XRS, Bio-Rad, USA). *Vibrio parahaemolyticus* VP20 was employed as a positive control.

Table 1. Primers used in the identification and virulence gene detection

Primer	Primer sequence (5′ → 3′)	Amplicon size (bp)	Reference
toxR-F	GTCTTCTGACGCAATCGTTG	368	[75]
toxR-R	ATACGAGTGGTTGCTGTCATG		
tdh-F	GTAAAGGTCTCTGACTTTTGGAC	269	[76]
tdh-R	TGGAATAGAACCTTCATCTTCACC		
trh-F	TTGGCTTCGATATTTTCAGTATCT	500	[76]
trh-R	CATAACAAACATATGCCCATTTCCG		

2.7 Antibiotic Susceptibility Test

The *V. parahaemolyticus* isolates were examined for susceptibilities against 14 antibiotics (Table 2). These antibiotics were selected based on their importance in the clinical setting and aquaculture industry. Quinolones (nalidixic acid and levofloxacin) and third-generation cephalosporin (cefotaxime and ceftazidime) are indicated in severe Vibriosis, whereas tetracycline is recommended in cholera[77–80]. The ampicillin-sulbactam covers bacterial infections that are resistant to ampicillin[81]. Carbapenem (imipenem) and aminoglycoside (amikacin, gentamicin and kanamycin) are broad-spectrum antibiotics used in infections caused by Gram-negative and Gram-positive bacteria[82–85]. The *V. parahaemolyticus* isolates were also tested against trimethoprim-sulfamethoxazole and chloramphenicol, which are used in *Pneumocystis jirovecii* pneumonia and eye infections resulting from methicillin-resistant *Staphylococcus aureus* (MRSA), respectively[86,87]. On the other hand, oxytetracycline is an important drug in the aquaculture industry[88].

The isolates were subjected to the antibiotic susceptibility test by the Kirby disc diffusion method[89]. This method has been employed in several studies investigating the antibiotic sensitivity in *V. parahaemolyticus* isolated from food samples[90,91]. All the isolates were revived in Tryptic Soy Broth (TSB) (HiMedia, India) supplemented with NaCl (2% w/v) and shaken at 37°C for 18 hours at 200 RPM. After the incubation, 100 µL of the suspension was swabbed uniformly onto the surface of the Mueller-Hinton (HiMedia, India) agar plate with a sterile cotton swab. The agar plates were then incubated at 37°C for 18 hours. The diameter of the inhibition zones was measured and interpreted according to the

guidelines provided by Clinical and Laboratory Standards Institute M45[92]. The MAR index was calculated using the formula below, which was initially developed by Krumperman [93].

$$Multiple\ antibiotic\ resistance\ (MAR) = \frac{a}{b} = \frac{Number\ of\ antibiotics\ that\ the\ isolates\ are\ resistant\ to}{Total\ number\ of\ antibiotics\ the\ isolates\ are\ exposed\ to}$$

Table 2. The list of antibiotics tested in this study[94–96]

Class of antibiotics	Antibiotics	Concentration (µg)
Penicillin	Ampicillin	10
β-lactamase inhibitor	Ampicillin-sulbactam	30
Third-generation cephalosporin	Cefotaxime	30
	Ceftazidime	30
Carbapenem	Imipenem	10
Aminoglycoside	Amikacin	30
	Gentamicin	30
	Kanamycin	30
Dihydrofolate reductase inhibitor	Trimethoprim-sulfamethoxazole	25
Tetracycline	Tetracycline	30
	Oxytetracycline	30
Quinolone	Nalidixic acid	30
	Levofloxacin	5
Anti-50S antimicrobial	Chloramphenicol	30

2.8 Plasmid Profiling

The bacterial isolates were revived in Tryptic Soy Broth (TSB) (HiMedia, India) supplemented with NaCl (2% w/v) and shaken at 37°C in a shaker incubator (200 RPM) for 18 hours. 1.5 mL of the suspension were transferred into a new microcentrifuge tube, and this was used for the plasmid extraction using the GF-1 Plasmid DNA Extraction Kit (Vivantis Technologies, Malaysia). The plasmid DNA was separated by gel electrophoresis in 1.0% agarose gel and visualised under UV light with a gel documentation system (ChemiDoc^{TM} XRS, Bio-Rad, USA).

2.9 Statistical Analysis

The analysis of the data was conducted using the statistical analysis software, IBM® SPSS® Statistics version 26. A one-way ANOVA test was performed to determine if the difference between the mean *Vibrio* count in the four types of shellfish samples were statistically significant. Besides that, a chi-squared test was used to determine whether the differences in the prevalence of *Vibrio parahaemolyticus* in seafood from wet markets and supermarkets were statistically different. Another chi-squared test was employed to study the relationship between different types of establishments and the number of *V. parahaemolyticus* isolates with a MAR index of more than 0.2. A difference was considered statistically significant when $p < 0.05$.

3. Results

3.1 Microbial Load of total Vibrio species

A total of 77 shellfish comprising of short-necked clam (*Paratapes undulatus*) (n = 19), blood clam (*Tegillarca granosa*) (n = 20), tiger prawn (*Penaeus monodon*) (n = 19) and Indian white shrimp (*Penaeus indicus*) (n = 19) were sampled from two wet markets and two supermarkets in Selangor, Malaysia.

The mean *Vibrio* count is calculated according to the sample type from the respective sampling sites (Table 3). Out of 16 groups of shellfish samples, 14 groups were contaminated with more than 5 log CFU/g of *Vibrio* species, which is the minimum bacterial load required to cause symptoms of Vibriosis in human hosts[61]. The only group of samples with a mean *Vibrio* count lower than the infectivity limit are tiger prawns from Wet market 1 and Indian white shrimps from Supermarket 2. Overall, the mean density of *Vibrio* species in all the shellfish samples ranges from 4.66 ± 0.40 to 8.95 ± 0.00 log CFU/g. The highest count is seen in short-necked clams from Wet market 2, followed by blood clams from Wet market 1 (6.06 ± 0.01 log CFU/g) and short-necked clams from Wet market 1 (5.98 ± 0.04). The lowest *Vibrio* count is detected in tiger prawns from Wet market 1.

The highest mean *Vibrio* count in each seafood sample type from all the markets, 6.52 log CFU/g, is seen in short-necked clam samples, followed by 5.77 log CFU/g in blood clams, 5.50 log CFU/g in Indian white shrimps and 5.34 log CFU/g in tiger prawns. One-way ANOVA test performed showed a significant difference between the mean of *Vibrio* count in the four types of shellfish (p = 0.005). Mean *Vibrio* count in Indian white shrimps is significantly lower than short-necked clams (-1.03, 95% CI = -1.95 - -0.10, p = 0.022). Besides that, tiger prawns also have mean *Vibrio* count that is significantly lower than short-necked clams (-1.19, 95% CI = -2.11 - -0.27, p = 0.005). However, the mean *Vibrio* count was not statistical significant between the blood clam and short-necked clam samples (- 0.76, 95% CI = -1.68 - 0.17, p = 0.005).

Table 3. Mean total *Vibrio* species count listed according to the type of seafood samples and sampling sites.

Sample	Mean of total *Vibrio* count (log CFU/g) Mean ± Standard deviation			
	Wet market 1	**Wet market 2**	**Supermarket 1**	**Supermarket 2**
Paratapes undulatus (Short-necked clam)	5.98 ± 0.04	8.95 ± 0.00	5.71 ± 0.17	5.46 ± 0.08
Tegillarca granosa (Blood clam)	6.06 ± 0.01	5.75 ± 0.02	5.42 ± 0.03	5.84 ± 0.03
Penaeus monodon (Tiger prawn)	4.66 ± 0.40	5.79 ± 0.05	5.21 ± 0.08	5.67 ± 0.05
Penaeus indicus (Indian white shrimp)	5.41 ± 0.11	5.74 ± 0.05	4.89 ± 0.21	5.95 ± 0.04

3.2 Molecular Identification of Vibrio parahaemolyticus

The PCR assay revealed positive amplification of the *toxR* gene with 368 bp amplicon band in 55.8% (43/77) of the presumptive *Vibrio parahaemolyticus* isolates (Table 4). The incidence of *Vibrio parahaemolyticus* in the shellfish samples was highest in samples collected from Supermarket 1 at 70.0% (14/20), followed by Wet market 1 at 61.0% (11/18). Supermarket 1 presented the lowest prevalence of *V. parahaemolyticus* at 35% (7/20). Among the samples, *V. parahaemolyticus* is most prevalent in tiger prawn 78.9% (15/19), followed by Indian white shrimp 68.4% (13/19), blood clam 45.0% (9/20) and short-necked clam 31.6% (6/19) (Table 2). The results of a chi-squared test performed showed no significant association between the number of *toxR*-positive *V. parahaemolyticus* isolates in shellfish from wet markets and supermarkets (51.2% vs. 48.8%, $p = 0.539$). None of the 43 *V. parahaemolyticus* isolates harboured the thermostable direct haemolysin (*tdh*) and thermostable direct haemolysin-related (*trh*) virulence genes.

Table 4. Prevalence of *Vibrio parahaemolyticus* in the shellfish samples

Sites	**Wet market 1**		**Wet market 2**		**Supermarket 1**		**Supermarket 2**		**Overall**	
Type of shellfish	Total no. of isolates	*toxR*-positive (%)	Total no. of isolates	*toxR*-positive (%)	Total no. of isolates	*toxR*-positive (%)	Total no. of isolates	*toxR*-positive (%)	Total no. of isolates	*toxR*-positive (%)
Paratapes undulatus (Short-necked clam)	5	1 (20.0)	4	1 (25.0)	5	3 (60.0)	5	1 (20.0)	19	6 (31.6)
Tegillarca granosa (Blood clam)	5	4 (80.0)	5	2 (40.0)	5	0 (0.0)	5	3 (60.0)	20	9 (45.0)
Penaeus monodon (Tiger prawn)	3	3 (100)	6	4 (67.0)	5	3 (60.0)	5	5 (100)	19	15 (78.9)
Penaeus indicus (Indian white shrimp)	5	3 (60.0)	4	4 (100)	5	1 (20.0)	5	5 (100)	19	13 (68.4)
Total	**18**	**11 (61.0)**	**19**	**11 (58.0)**	**20**	**7 (35.0)**	**20**	**14 (70.0)**	**77**	**43 (55.8)**

3.3 Antibiotic Susceptibility Test and Multiple Antibiotic Resistance (MAR) Indices

The antibiotic resistance profiles of all 43 *V. parahaemolyticus* isolates are summarised in Table 5. A vast majority of the bacterial isolates were resistant to ampicillin (95.3%). Similarly, resistance towards the combination antibiotic, ampicillin-sulbactam was also high (81.4%). The antibiotic resistance profile of the *V. parahaemolyticus* isolates towards third-generation cephalosporins showed mixed results. There was nearly an equal distribution between isolates that were resistant (37.2%) to cefotaxime and sensitive (34.5%) towards the said drug. In contrast, most isolates were susceptible to ceftazidime (55.8%), with a high proportion of the isolates showing intermediate resistance towards this antibiotic (37.2%). Although most of the *V. parahaemolyticus* isolates (76.7%) were susceptible to the carbapenem antibiotic, imipenem, 23.3% (10/43) were resistant to this antibiotic. This

finding is worrying because carbapenems are broad-spectrum antibiotics commonly used as the last line of drug in Gram-negative and Gram-positive bacterial infections[82,83].

Table 5. Antibiotic resistance profile of the *Vibrio parahaemolyticus* isolated from the shellfish.

Antibiotics	**Resistant** n **(%)**	**Intermediate** n **(%)**	**Sensitive** n **(%)**
Ampicillin 10µg	41 (95.3)	0 (0.0)	2 (4.7)
Ampicillin-sulbactam 30µg	35 (81.4)	5 (11.6)	3 (7.0)
Cefotaxime 30µg	16 (37.2)	12 (27.9)	15 (34.9)
Ceftazidime 30µg	3 (7.0)	16 (37.2)	24 (55.8)
Imipenem 10µg	10 (23.3)	0 (0.0)	33 (76.7)
Amikacin 30µg	1 (2.3)	3 (7.0)	39 (90.7)
Gentamicin 30µg	2 (4.7)	1 (2.3)	40 (93.0)
Kanamycin 30µg	4 (9.3)	9 (20.9)	30 (69.8)
Trimethoprim-sulfamethoxazole 25µg	0 (0.0)	0 (0.0)	43 (100.0)
Tetracycline 30µg	8 (18.6)	3 (7.0)	32 (74.4)
Oxytetracycline 30µg	7 (16.3)	1 (2.3)	35 (81.4)
Nalidixic acid 30µg	1 (2.3)	1 (2.3)	41 (95.3)
Levofloxacin 5µg	1 (2.3)	0 (0.0)	42 (97.7)
Chlorampenicol 30µg	0 (0.0)	0 (0.0)	43 (100.0)

In contrast, the *V. parahaemolyticus* isolates were highly susceptible to quinolones (levofloxacin, 97.7%; nalidixic acid, 95.3%), aminoglycosides (gentamicin, 93%; amikacin, 90.7%; kanamycin, 69.8%) and tetracyclines (oxytetracycline, 81.4%; tetracycline, 74.4%). Unsurprisingly, all the isolates were sensitive towards trimethoprim-sulfamethoxazole and chloramphenicol.

The isolates exhibited MAR indices ranging from 0.07 to 0.36. The highest MAR index (0.36) was seen in 5/43 (7.0%) bacterial isolates. These isolates were resistant to penicillin, beta-lactamase inhibitors, third-generation cephalosporins, carbapenems and, either quinolones or tetracyclines. The highest frequency of the MAR index was 0.21, followed by 0.29. 37.2% (16/43) of the *V. parahaemolyticus* isolates exhibited a MAR index of 0.21, whereas 25.9% (11/43) had a MAR index of 0.29. Among all 43 *V. parahaemolyticus* isolates, only one (2.3%) did not exhibit MAR as it was sensitive to all the antibiotics included in the study. Interestingly, all 10 *V. parahaemolyticus* isolates that were resistant to

imipenem demonstrated MAR indices ranging from 0.21 to 0.36, which is the highest MAR index reported in this study.

Bacterial isolate with a MAR index of more than 0.2 connotes the origin of the isolate from a high-risk source of contamination. This means that a particular strain of *Vibrio parahaemolyticus* was exposed to an increased number of antimicrobials[97,98]. In the present study, 30/43 *V. parahaemolyticus* isolates had a MAR index of more than 0.2. Cumulatively, this was expressed by 69.8% of the bacterial isolates which were resistant to 3 to 5 different antibiotics tested. Of the 30 *V. parahaemolyticus* isolates with a MAR index exceeding 0.2, an equal number of bacterial strains were isolated from wet market and supermarket samples. A chi-squared test was performed to study the relationship between different types of establishments and the risk of contamination. The analysis shows that there was no significant difference in the risk of contamination among different sources of shellfish (50% vs. 50%; $p = 0.054$).

3.4 Plasmid Profiles of Vibrio parahaemolyticus

All 43 *toxR*-positive *V. parahaemolyticus* isolates were subjected to plasmid profiling and the profiles are illustrated in Table 6. Among all the *V. parahaemolyticus* isolates, 18 (41.9%) isolates harboured at least one plasmid, whereas the majority did not possess any plasmids (25/43; 58.1%). Interestingly, all the isolates without plasmids (excluding VV34) were resistant to at least 1 antibiotic with MAR indices ranging from 0.07 to 0.29 (Table 7).

Cumulatively, the number of plasmids in the *V. parahaemolyticus* isolates ranges from one to four, whereby 20.9% (9/43), 14.0% (6/43), and 4.7% (2/23) of the isolates had one, two and three plasmids, respectively. Only one (2.3%) strain of *V. parahaemolyticus* harboured four plasmid DNA bands with molecular weights of 1.5 kb, 5.2 kb, 7 kb and above 10 kb. This *V. parahaemolyticus* isolate, VV40, was resistant to four antibiotics which are ampicillin, ampicillin-sulbactam, cefotaxime and imipenem. In contrast, the VV34 strain, which was susceptible to all the antibiotics tested, did not harbour any plasmids.

The molecular weight of the plasmids that reside in the *V. parahaemolyticus* isolates ranges from 1 kb to more than 10 kb. The plasmid DNA bands in each strain of bacteria were categorised according to the number of plasmids and plasmid sizes. With that, 14 different plasmid patterns were encountered among the *V. parahaemolyticus* isolates. The most common plasmid pattern seen is 1.1, which refers to the possession of one plasmid with a size of more than 10 kb. This pattern has been identified in 4/43 (9.3%) *V. parahaemolyticus* isolates.

Besides that, VV09, VV30 and VV37, which were resistant to five out of 14 antibiotics tested, had relatively bigger plasmids. VV37 has only one plasmid that is more than 10 kb but is resistant to five different antibiotics, namely, ampicillin, ampicillin-sulbactam, cefotaxime, imipenem, and tetracycline. VV30 has plasmids with the molecular weight of more than 10 kb and 10 kb, whereas VV09 has 10 kb- and 2kb-sized plasmids. Strikingly, 9 out of 10 (90%) *Vibrio parahaemolyticus* isolates that were resistant to imipenem had one to four plasmids, sizes ranging from 1.5 kb to more than 10 kb. VV38 is the only bacterial strain that was resistant to imipenem but did not harbour any plasmids.

Table 6. Plasmid profiles of the *Vibrio parahaemolyticus* isolates from shellfish samples

Number of plasmids	**Plasmid pattern**	**Plasmid size** (kb)	**Number of isolates** (%)	***Vibrio parahaemolyticus* isolates**
0			25 (58.1)	VV02, VV10, VV11, VV12, VV21, VV22, VV23, VV25, VV26, VV27, VV34, VV38, VV39, VV41, VV42, VV43, VV52, VV56, VV57, VV58, VV65, VV68, VV69, VV70, VV75
1	1.1	>10	4 (9.3)	VV37, VV45, VV48, VV49
	1.2	10	2 (4.7)	VV29, VV67
	1.3	7	1 (2.3)	VV19
	1.4	5.2	1 (2.3)	VV64
	1.5	1.5	1 (2.3)	VV71
2	2.1	>10, 10	1 (2.3)	VV30
	2.2	10, 4	1 (2.3)	VV14
	2.3	10, 2	1 (2.3)	VV09
	2.4	7, 6.2	1 (2.3)	VV07
	2.5	7, 4	1 (2.3)	VV24
	2.6	6.2, 5.2	1 (2.3)	VV01
3	3.1	10, 6.2, 2.5	1 (2.3)	VV08
	3.2	10, 5.2, 2	1 (2.3)	VV35
4	4.1	>10, 7, 5.2, 1.5	1 (2.3)	VV40

Table 7. Plasmid profiles and antibiograms of *Vibrio parahaemolyticus* isolates.

Number of plasmids	Plasmid pattern	Plasmid size (kb)	MAR index	Antibiogram	Isolates
0			0.0		VV34
			0.07	AMP	VV22, VV39
			0.14	AMP/SAM	VV02, VV26, VV52, VV57, VV65
			0.14	AMP/OT	VV43
			0.21	AMP/SAM/TE	VV12, VV23
			0.21	AMP/CAZ/K	VV10
			0.21	AMP/SAM/K	VV11
			0.21	AMP/CTX/K	VV21
			0.21	AMP/AK/K	VV25
			0.21	AMP/IPM/OT	VV38
			0.21	AMP/SAM/OT	VV41
			0.21	AMP/SAM/TE	VV68
			0.21	SAM/CTX/TE	VV69
			0.21	AMP/SAM/CTX	VV75
			0.29	AMP/SAM/CTX/OT	VV27, VV42, VV70
			0.29	AMP/SAM/TE/OT	VV56
			0.29	AMP/SAM/CTX/IPM	VV58
1	1.1	> 10	0.21	AMP/SAM/CN	VV49
			0.29	AMP/SAM/IPM/OT	VV45
			0.29	AMP/SAM/CTX/IPM	VV48
			0.36	AMP/SAM/CTX/IPM/TE	VV37
	1.2	10	0.14	AMP/SAM	VV67
	1.2	10	0.29	AMP/SAM/CTX/CN	VV29
	1.3	7	0.14	AMP/SAM	VV19
	1.4	5.2	0.21	AMP/SAM/IPM	VV64
	1.5	1.5	0.21	AMP/SAM/CTX	VV71

Number of plasmids	Plasmid pattern	Plasmid size (kb)	MAR index	Antibiogram	Isolates
2	2.1	> 10, 10	0.36	AMP/SAM/CAZ/IPM/TE	VV30
	2.2	10, 4	0.14	AMP/SAM	VV14
	2.3	10, 2	0.36	AMP/SAM/CAZ/IPM/NA	VV09
	2.4	7, 6.2	0.21	AMP/SAM/CTX	VV07
	2.5	7, 4	0.14	AMP/SAM	VV24
	2.6	6.2, 5.2	0.29	AMP/SAM/CTX/IPM	VV01
3	3.1	10, 6.2, 2.5	0.21	AMP/SAM/CTX	VV08
	3.2	10, 5.2, 2	0.29	AMP/SAM/CTX/TE	VV35
4	4.1	> 10, 7, 5.2, 1.5	0.29	AMP/SAM/CTX/IPM	VV40

AMP (Ampicillin), SAM (Ampicillin-sulbactam), CAZ (Ceftazidime), CTX (Cefotaxime), IPM (Imipenem), AK (Amikacin), CN (Gentamicin), K (Kanamycin), TE (Tetracycline), OT (Oxytetracycline), NA (Nalidixic acid), LEV (Levofloxacin), SXT (Trimethoprim-sulfamethoxazole), C (Chloramphenicol)

4. Discussion

The *Vibrio* species are commonly found in marine environments, living in the water, sediments, plankton, aquatic animals, and flora[1,38,49,99]. Therefore, it is essential to continuously monitor the density of these pathogens to ensure the safety of seafood. The microbial load of *Vibrio* species in the shellfish sampled in this study is reported in Table 3. In this study, *Vibrio* species were recovered from all the seafood sampled from all four sampling locations. The mean total count of *Vibrio* species ranged from 4.66 ± 0.40 to 8.95 ± 0.00 log CFU/g. As of now, the microbiological limits of *V. parahaemolyticus* count in seafood is not regulated in Malaysia[88]. However, Letchumanan and colleagues suggested that a minimum of 5 log CFU/g of *Vibrio* count is required to cause symptoms of Vibriosis in human hosts[61]. In the present study, 14/16 groups of shellfish samples listed in Table 3 were contaminated with more than 5 log CFU/g of *Vibrio* species. Considering this, groups of shellfish that harboured a mean *Vibrio* count of more than 5 log CFU/g can potentially become a health hazard to humans.

The highest mean total *Vibrio* count detected in this study was higher than the microbial load of *Vibrio* species reported by other studies conducted on seafood. In the study done by Letchumanan *et al.*[61] in Malaysia, the mud crabs (*Scylla serrate*), flower crabs (*Portunus pelagicus*), carpet clams (*Paphia textile*) and hard shell clams (*Meretrix meretrix*)

contained a microbial load of 2.45 to 6.63 log CFU/g. Another study done by the same author reported the microbial load of *Vibrio* species ranging from 4.36 log CFU/mL to 6.34 log CFU/mL in the prawns sampled from Malaysia[74]. It is also noteworthy that the findings of this study are higher than the results obtained by Lamon *et al.*[100] in Italy. The mean *Vibrio* count in the Mediterranean mussel (*Mytilus galloprovincialis*) and grooved carpet shell (*Ruditapes decussatus*) sampled in the study were well below 5 log CFU/g before the purification process[100]. The upward trend in the mean concentration of *Vibrio* species calls for the attention of the public health body to monitor the levels of this enteropathogen closely in seafood, especially in shellfish.

High levels of *Vibrio* species have been detected in many types of seafood samples from this region[61,74,88]. This can be attributed to the ubiquitous existence of these organisms in estuaries and coastal regions compounded by the hot climate in Malaysia, which promotes the growth of *Vibrio* species[1,38,49,88,99]. Nevertheless, this study has shown that the mean count of *Vibrio* species was significantly different among the four species of shellfish ($p < 0.05$). The mean *Vibrio* count in short-necked clams was significantly higher than in both species of shrimps analysed in this study. To our best knowledge, the present study is the first to statistically analyse the difference in the microbial load of *Vibrio* species among different types of shellfish.

Despite the increased risk of contamination associated with all types of shellfish, the higher density of *Vibrio* species in short-necked clams can be linked to the filter-feeding habits of these molluscs. Bivalves such as the short-necked clams feed by filtering water through their gills, allowing bacteria and other contaminants to mobilise and concentrate in the digestive tract[13,32,101,102]. However, the findings cannot be extrapolated to all types of bivalve molluscs. This is because the microbial load of *Vibrio* species in the blood clams sampled in this study were not significantly higher than both species of crustaceans, disallowing a general correlation to be formed. Nonetheless, the findings of this study indicate that all shellfish are potential vehicles of transmission of the *Vibrio* species. The high risk of contamination directly translates into a high risk of gastroenteritis related to the consumption of crustaceans and bivalve molluscs. To avert the incidence of Vibriosis, Centers for Disease Control and Prevention (CDC) recommends boiling or steaming shellfish for an appropriate duration of time[78]. As evidenced by Liu *et al.*[103], the direct exposure of shellfish to high temperatures has been shown to reduce the concentrations of the pathogen to undetectable levels (< 3 MPN/g), consequently ensuring safe consumption of seafood.

The *toxR* gene is commonly selected as the target gene in the detection of *Vibrio parahaemolyticus* in seafood[10,60,74,88,104]. This is because the *toxR*-PCR assay is highly

accurate attributable to the heterogeneity in the nucleotide sequence of *toxR* gene among the species in the *Vibrio* genus[105,106]. A total of 43/77 (55.8%) presumptive *V. parahaemolyticus* isolates from the shellfish samples were positive for the *toxR* gene, confirming the presence of the pathogen. Tiger prawn was detected at the highest incidence rate (78.9%), followed by Indian white shrimp (68.4%), blood clam (45.0%) and short-necked clam (31.6%). The high prevalence of *V. parahaemolyticus* in these shellfish samples can be attributed to the temperature of seawater in tropical countries like Malaysia, which provides a conducive environment for *V. parahaemolyticus* to survive and grow in seafood[1,10,104,107]. Interestingly, the highest prevalence of *V. parahaemolyticus* was found in tiger prawns, which contained the lowest mean *Vibrio* count among all the shellfish samples. Conversely, the short-necked clams with the lowest incidence of the pathogen possessed the highest microbial load of *Vibrio* species. These findings are in line with previous studies[98,108], which supports the use of PCR amplification over the direct plating method in identifying *V. parahaemolyticus* as the molecular detection technique has shown to produce more accurate results than the latter.

There is a considerable amount of literature concerning *V. parahaemolyticus* in seafood from Asia. Despite the subtle variation in the methodologies employed, ultimately, most studies have consistently used the *toxR*- or *tlh*-PCR amplification technique, which allows accurate comparisons of findings to be made[90,109–113]. The overall prevalence of *V. parahaemolyticus* in the shellfish sampled in this study is far lower compared to most studies done in other tropical and subtropical countries. In Malaysia, Narayanan *et al.*[90] reported that 85.71% (120/140) of blood clam (*Anadara granosa*), shrimp (*Penaeus* species), surf clam (*Paphia undulata*) and squid (*Loligo* species) were positive for *V. parahaemolyticus*. Besides that, Narayanan *et al.*[90] found that 96.8% (31/32) of the shellfish from Kerala, India, were contaminated with the pathogen. In Bangladesh, 69.44% of the shrimps harvested from farms contained *V. parahaemolyticus*, as outlined by Siddique *et al.*[110]. In East Asia, recent studies showed a varying incidence of the pathogen in shellfish. The occurrence of *V. parahaemolyticus* was reported as 20.0% and 14.0% in short-necked clams (*Paphia variegata*) and white shrimps (*Penaeus vannamei*) from China, which is lower than our findings[111]. In contrast, oysters (*Crassostrea gigas*) sampled in Kang *et al.*[113] showed a higher prevalence (85.5%) of the bacteria in their study.

In the present study, we found that the occurrence of *V. parahaemolyticus* was slightly higher in wet market samples as compared to the supermarket samples (51.2% vs. 48.8%). To our knowledge, this is the first study to show that the prevalence of *V. parahaemolyticus* was not significantly associated with the type of establishments the shellfish are obtained from ($p > 0.05$). Unlike the present study, Tan *et al.*[88] demonstrated a significantly lower

prevalence of *V. parahaemolyticus* in short mackerels (*Rastrelliger bachysoma*) from hypermarkets compared to mini market and wet markets samples (83.3% vs. 89.1% vs. 95.2%; $p < 0.05$). In contrast, the study by Lee *et al.*[98] reported a lower prevalence of the pathogen in wet market fish samples compared to fishes from the supermarket (47% vs. 53%). The mixed evidence on the contamination rate of shellfish in different establishments supports the idea outlined by previous studies[61,114]. These studies suggested that cross-contamination can take place at any point during handling, preparing, processing, transporting, and storing of shellfish, irrespective of the sampling site[61,114]. If the cold chain is broken, multiplication of *V. parahaemolyticus* cells can occur as a result of the exposure of shellfish to room temperatures[115]. Therefore, temperature control plays a vital role in the preservation of shellfish. Liu *et al.*[103] and Vasudevan *et al.*[116] have highlighted the importance of freezing in maintaining the freshness of shellfish up to the point of human consumption. Ice crystals that form during frozen storage disrupt bacterial cell structures, consequently terminating viable *V. parahaemolyticus* cells in shellfish[103].

Historically, the two well-defined haemolysins encoded by the *tdh* and *trh* genes are more frequently found in *V. parahaemolyticus* isolated from clinical samples than environmental and food samples[98,110,117]. Therefore, these putative genes provide the most discriminatory ability to detect pathogenic strains of *V. parahaemolyticus*[40,41]. Generally, the occurrence of pathogenic strains of *V. parahaemolyticus* depends on the isolation rate of the bacteria in seafood samples. As stated in the previous section, the prevalence of foodborne pathogens varies according to different geographical sites and species of seafood. Therefore, direct comparisons of the incidence of *tdh*- and *trh*-positive *V. parahaemolyticus* among individual studies should be interpreted cautiously.

Although a high prevalence of *V. parahaemolyticus* was detected in the shellfish samples, none of the *V. parahaemolyticus* strains was positive for *tdh* and *trh* genes. This shows that the expression of TDH and TRH does not exist among the *V. parahaemolyticus* strains isolated from the shellfish sampled from the wet markets and supermarkets in Selangor. The findings of our study are in agreement with several studies undertaken around this region. A study by Narayanan *et al.*[90] demonstrated that all 140 samples of the bivalve molluscs and crustaceans obtained from Selangor did not present with either *tdh* or *trh* genes. Similar results were also found in the study conducted by Kang *et al.*[113], whereby none of the oysters (*Crassostrea gigas*) was contaminated with *tdh*- or *trh*-positive strains of *Vibrio parahaemolyticus*.

Previous studies performed in Asia have demonstrated a low occurrence of the *tdh* and *trh* in the *V. parahaemolyticus* isolates from seafood. In Lee *et al.*[98], 4 out of 165 (2.4%) *toxR*-positive *V. parahaemolyticus* isolates from fish samples were positive for *trh* gene, whereas none of the isolates expressed the *tdh*. The oysters (*Crassostrea gigas*) sampled in South Korea were contaminated with 9.1% of *trh*-positive *Vibrio parahaemolyticus*. Contrastingly, none of the isolates was positive for *tdh*[118]. Hu *et al.*[111] demonstrated that the 2.6% (2/77) and 1.3% (1/77) of *V. parahaemolyticus* isolated from shellfish samples from China were positive for *tdh* and *trh* gene, respectively. Despite the low prevalence of pathogenic strains of the bacteria reported in most studies, a minority of the studies conducted have illustrated a higher incidence of pathogenic *V. parahaemolyticus* strains in seafood. For instance, 21.95% of shrimp and 18.75% of fish samples from China were contaminated with *trh*-positive *Vibrio parahaemolyticus*, whereas the prevalence of *tdh* was 7.32% and 15.63% in shrimps and fish, respectively[119]. As outlined by Raghunath[41], the vast difference in the occurrence of *tdh*- and *trh*-positive strains demonstrated by individual studies is attributable to the different sampling sites, source of shellfish and detection method of the bacteria.

There is still insufficient data to conclude that all the *V. parahaemolyticus* strains in this present study are non-pathogenic, based entirely on the absence of *tdh* and *trh* genes. This is because several other mechanisms are involved in the mediation of virulence in this pathogen, such as the *tlh*, T3SS, T6SS, biofilm, siderophore and protease production[15,120–123]. Besides that, the demonstration of β-haemolytic action, which is highly specific to *tdh*, was recently seen in *tdh and* trh-negative strains of *Vibrio parahaemolyticus*[90,124]. This raises questions on the involvement of mechanisms apart from the *tdh*, the Kanagawa phenomenon (KP) action of the bacteria[43,125,126]. Despite the absence of *tdh*- and *trh*-positive *V. parahaemolyticus* strains in the present study, continuous monitoring of the shellfish sold in this region is important to ensure the food safety of seafood products. However, it is imperative that future work targets other virulence factors to determine the pathogenicity of the *V. parahaemolyticus* strains in seafood.

The antibiotic susceptibility test was performed for all 43 *V. parahaemolyticus* isolates from the shellfish samples. The results show that nearly all of the bacterial isolates (95.3%) were resistant to ampicillin. However, resistance to β-lactams such as ampicillin is not uncommon in *V. parahaemolyticus*. Due to the misuse and overuse of ampicillin in aquaculture, high incidences of ampicillin-resistant *V. parahaemolyticus* have been reported extensively in literature worldwide[55,61,90,98,118,119]. The increasing trend in the minimum inhibition concentration (MIC) of ampicillin from 64 µg/mL in 2011 to 128 µg/mL in 2013, as outlined by Al-Othrubi *et al.*[60], have highlighted the worsening case of resistance against

this drug. Although ampicillin is not used in the management of Vibriosis, these findings are of great concern as it impedes the role of ampicillin in the empirical management of bacterial infections[55]. Besides that, most of the *V. parahaemolyticus* isolates were also resistant to ampicillin-sulbactam (81.4%). Alarmingly, this value is much higher than those reported by other studies in Malaysia[74,88,90]. Although the sample source and detection methods could have contributed to the differences, the resistance to β-lactam/β-lactamase inhibitor drug in this study should still be highlighted, considering the significance of the drug in treating infections caused by β-lactamase-containing *S. aureus*, *H. influenzae* and *E. coli*[81,127].

A notable resistance pattern was observed with cefotaxime (37.2%) in the present study. On the contrary, most of the *V. parahaemolyticus* isolates were found to be sensitive to ceftazidime (55.8%). Similar resistance patterns to third-generation cephalosporins were also demonstrated by other studies, including the study done by Narayanan *et al.*[90]. In Lee *et al.*[98], more than half (52%) of the *V. parahaemolyticus* isolates were resistant to cefotaxime, whereas 48% of these isolates exhibited resistance to ceftazidime. Meanwhile, Kang *et al.*[118] reported that most isolates (63.6%) from oysters (*Crassostrea gigas*) were sensitive to cefotaxime. This study also demonstrated that none of the *V. parahaemolyticus* strains was resistant to the aforementioned drug. The discrepancies in the literature regarding the resistance of *V. parahaemolyticus* to third-generation cephalosporins reflects on the antibiotic-prescribing practices in the local setting. However, the resistance to β-lactams like this group of antibiotics is a significant public health threat. The recent discoveries of extended-spectrum beta-lactamases (ESBL) - producing strains of *V. parahaemolyticus* in Korea and India raise concerns about the spread of these strains locally[128,129].

Carbapenems such as imipenem are proven effective in providing complete coverage in human infections resulting from ESBL-producing organisms[130,131]. Carbapenems are also frequently used as the drug of last resort in infections caused by Gram-positive and Gram-negative bacteria[82,83]. The over-reliance on carbapenems have therefore resulted in the increasing resistance to these group of drugs. This is evidenced by the 23.3% resistance rate to imipenem in the present study. These results tie well with similar studies done in this region. Lee *et al.*[98] have reported that 12.0% of the *V. parahaemolyticus* isolated from marine fish and freshwater fish were resistant to imipenem. Similarly, a low incidence (2%) of imipenem resistance was detected in *V. parahaemolyticus* isolated from banana prawns and red prawns from Malaysia[74]. Letchumanan *et al.*[61] showed that 0.5% out of 200 isolates from crustaceans and bivalve molluscs were resistant to the same drug. Contrary to these findings, Narayanan *et al.*[90] and Siddique *et al.*[110] found that all the *V. parahaemolyticus* isolates from the seafood samples were susceptible to carbapenems such as imipenem and

meropenem. The low resistance rates show that the global resistance to this group of antibiotics is still at the initial stage. Hence, immediate action is needed to preclude the further spread of carbapenem resistance among *V. parahaemolyticus* in seafood.

In the present study, high susceptibility rates to quinolones (levofloxacin, 97.7%; nalidixic acid, 95.3%) were seen. These findings are in line with other literature that has reported similar susceptibility patterns to quinolones[90,119]. The consistently high sensitivity of *V. parahaemolyticus* to quinolones reinforces the usage of these antimicrobials in the management of Vibriosis, thus reducing mortality rates caused by the disease[77,78]. The isolates in this study were highly susceptible to trimethoprim-sulfamethoxazole (100%), chloramphenicol (100%), aminoglycosides (gentamicin, 93%; amikacin, 90.7%; kanamycin, 69.8%) and tetracyclines (oxytetracycline, 81.4%; tetracycline, 74.4%). Similarly, a study reported high susceptibility of *V. parahaemolyticus* isolates to chloramphenicol (91.04%), tetracycline (83.58%), and aminoglycosides such as gentamicin (74.63%) and amikacin (65.67%)[88]. Several other studies have also found high sensitivity rates to these groups of antibiotics in *V. parahaemolyticus* from food sources[90,132].

In this study, the MAR index of the *V. parahaemolyticus* isolates ranges from 0.00 to 0.36. Three isolates exhibited resistance to five antibiotics, yielding the highest MAR index value seen in this study. Although the MAR index provides a good measure of the severity of antibiotic resistance in the samples, comparisons of MAR indices between studies are impossible to make due to the variation in the types of antibiotics tested and the total number of antibiotics used in individual studies. For example, Narayanan *et al.*[90] demonstrated that the MAR indices of *V. parahaemolyticus* isolates ranged from 0.00 to 0.71. The isolate with the highest MAR index was resistant to 17 out of 24 antibiotics. However, in Siddique *et al.*[110], the MAR index ranged from 0.07 to 0.27, and the highest MAR index was demonstrated by *V. parahaemolyticus* isolates that were resistant to four out of 15 antibiotics.

Bacterial isolates with a MAR index of more than 0.2 reflects the origin of the strain from contaminated sources such as aquaculture and agriculture farms[97,98]. The excessive usage of antibiotics in these sectors exerts selection pressure on the microflora in the water and soil, resulting in the growth of multi-drug resistant organisms[53,59,133]. Alternatively, isolates that have MAR indices less than 0.2, are thought to have originated from a low-risk source with lesser exposure to antibiotics[98]. In this study, 69.8% of the *V. parahaemolyticus* isolates had MAR indices of more than 0.2. A chi-squared test showed that the number of bacterial isolates with a MAR index of more than 0.2, was not significantly different between wet market and supermarket samples ($p = 0.054$)**.** This suggests that the shellfish from wet

markets and supermarkets had similar levels of exposure to antibiotics. Seafood samples from both locations are at equally high risk of transmitting multidrug resistant strains.

The advent and widespread usage of antibiotics for over 80 years have exerted selection pressure on bacteria such as *V. parahaemolyticus*. This has resulted in resistance against these therapeutic agents, as demonstrated in the present study. Antibiotics like ampicillin are excessively used in the agriculture industry and healthcare sectors[52]. Antimicrobials are used to prevent, treat and control diseases and promote the growth of marine products in the aquaculture industry[52,53]. In the healthcare setting, antibiotics are often used to treat self-limiting infections and indefinite diagnoses[52,134]. Ultimately, these anthropogenic activities involving the widespread usage of antibiotics have resulted in *V. parahaemolyticus* developing multi-drug resistance against common antibiotics[55]. The development of multi-drug resistance in *V. parahaemolyticus* is a public health and therapeutic concern. This is because these resistance genes can spread to human hosts through the consumption of contaminated seafood. Resistance genes can also be transmitted to human hosts via the lateral gene transfer of mobile genetic elements with resistance genes, from *V. parahaemolyticus* to other human pathogens[1,59].

Plasmids are one of the mobile genetic elements that contain important genes for bacterial survival, such as genetic elements encoding antibiotic resistance[135,136]. The selection pressure exerted by the frequent exposure to antibiotics promotes the transmission of plasmids with resistance genes among bacterial cells[53,59,133]. Therefore, profiling plasmids in bacteria like *V. parahaemolyticus* provides a better understanding of the mediation of antibiotic resistance in these bacteria. In this study, 18 out of 43 (41.9%) *V. parahaemolyticus* isolates possess one to four plasmid DNA bands, ranging from 1 kb to more than 10 kb in size. These findings are in line with other studies that have detected plasmid DNA in *V. parahaemolyticus* isolated from seafood[61,98,137].

However, the majority of the bacterial isolates in the present study did not harbour any plasmid. Based on the plasmid profiles of the isolates mentioned, 25/43 *V. parahaemolyticus* isolates (58.1%) were devoid of any plasmids. Interestingly, all except one of the bacterial strains (VV34) without plasmid were resistant to at least one antibiotic tested in this study. It can be postulated that the antibiotic resistance in these *V. parahaemolyticus* isolates was intrinsically mediated via chromosomes. Since *V. parahaemolyticus* exists ubiquitously in aquatic environments, they are constantly exposed to residues of antibiotics used in aquaculture, farming, and healthcare sectors. Antibiotics used in these economic sectors are often released into wastewater treatment plants[138]. The residual antibiotics in wastewater, compounded by antibiotics used in aquaculture, promote the growth of resistant

V. parahaemolyticus via the mutation of genes that control the activity of antibiotics in the cell[53,59,133,139]. Once a resistant mutant emerges, the antibiotic terminates strains sensitive to the antibiotic. Ultimately, this allows the resistant *V. parahaemolyticus* to thrive in the marine environment[139]. The findings of our study are in strong agreement with previous studies which have reported chromosomal mediation of antibiotic resistance in their respective studies[61,98,140].

The plasmid profiles of the *V. parahaemolyticus* isolates from the shellfish samples showed that the isolates that were resistant to the highest number of antibiotics tested (5/14) had relatively larger plasmids. Therefore, we cannot rule out the possibility of a relationship between plasmid sizes and the number of antibiotic resistance genes possessed by a bacterium. It is worth noting that in most cases, larger plasmids in Gram-negative bacteria are conjugative plasmids. These plasmid DNA bands possess a higher number of DNA base pairs which codes for the conjugation function of the bacteria[141]. Another striking pattern encountered in this study is the possession of at least one plasmid in most of *Vibrio parahaemolyticus* isolates (9/10; 90%) that were resistant to imipenem. This suggests the possibility of the imipenem resistance being acquired through laterally transferable plasmids that encode for carbapenemase genes[142,143]. However, existing studies have only described the chromosomal mediation of carbapenem resistance in *V. parahaemolyticus*[98,144]. Therefore, further analysis through plasmid curing assay is required to determine the role of plasmids in the antibiotic resistance phenotype of the *V. parahaemolyticus* isolates in this study[145].

There are several potential non-antibiotic methods that been effective against *V. parahaemolyticus* and dealing with antibiotic-resistant strains. Recently, bacteriophages or phage therapy has regained renewed interest in controlling Vibriosis and multidrug-resistant bacteria[80,146,147]. These phages are host specific, induces bacteriolysis and immediate counter action, readily isolated, cost effective, and generate less adverse effect compared to antibiotics[148–150]. The use of phage therapy in the aquaculture sector will eventually reduce the dependency for antibiotics and will allow the bacteria strains to lose their resistance traits[151–153].

Besides, recent literatures have been evidence in bioprospecting for natural products derived from plants[154,155], microbial origins[156–158] or animals with potential antimicrobial properties to fight against multidrug *Vibrio* strains[159–161]. *Streptomyces* sp., a soil derived bacteria has exhibited valuable properties to be biocontrol agent of *Vibrio*[150,162–165] and as probiotic in aquculture or animal husbandry[166–170]. These natural bacteria are safer and does not couse any resistance traits similar to antibiotics. Hence, the application of phage therapy

and *Streptomyces* sp. probiotics should be introduced in aquaculture sectors as a tool agaisnt bacterial infections and reduce the dependency towards antibiotics.

5. Conclusion

In summary, high densities of *Vibrio* species (> 5 log CFU/g) were found in 14 out of 16 groups of shellfish, increasing the risk of foodborne infection in human hosts. While most of the seafood analysed (55.8%) were contaminated with *V. parahaemolyticus*, none of the isolates harboured the genes encoding for TDH and TRH. This shows that all the strains of the bacteria are non-pathogenic. However, the complexity in the virulence mechanism of this bacteria calls for future studies to explore other factors facilitating the pathogenicity of *V. parahaemolyticus*. Besides that, the bacterial isolates exhibited resistance to different antibiotics. High resistance rates were seen towards common antibiotics such as ampicillin, ampicillin-sulbactam, cefotaxime and imipenem. The seriousness of antibiotic resistance is also reflected in the high proportion (69.8%) of *V. parahaemolyticus* isolates exhibiting a MAR index of more than 0.2. This is a threat to public health because the transmission of antibiotic resistance genes can occur via the lateral transfer of plasmids among bacterial cells. Since 41.9% of the *V. parahaemolyticus* isolates were shown to have plasmid DNAs, it is imperative to take immediate action to curb multi-drug resistance in *V. parahaemolyticus*. The relevant agencies should closely monitor and control the use of antibiotics in aquaculture, aqriculture and animal husbandry.

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