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Determination of antibiotic resistance patterns of *Vibrio parahaemolyticus* from shrimp and shellfish in Selangor, Malaysia

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Abstract : High consumer demand for seafood has led to the need for large-scale, reliable supply through aquaculture farming. However, bacterial infections - which can spread rapidly among the dense farming area pose a major threat to this industry. The farmers therefore often resort to extensive use of antibiotics, both prophylactically and therapeutically, in order to protect their stocks. The extensive use of antibiotics in aquaculture has been postulated to represent a major contributing factor in the rising incidence of antimicrobial resistant pathogenic bacteria in seafood; which may then lead to the spread of antimicrobial resistant bacteria in the environment as well as posing a significant threat to human health. This study aimed to characterize antibiotic resistance of *Vibrio parahaemolyticus* from shrimp and shellfish in Selangor, Malaysia. The antibiotic susceptibility of 385 *V. parahaemolyticus* isolates was investigated against 14 antibiotics followed by plasmid profiling and plasmid curing to determine the antibiotic mediation. A large number of isolates showed resistance to ampicillin (85%), amikacin (66.8%), and kanamycin (50.1%). A notable resistance pattern was also observed to the third generation cephalosporins (cefotaxime 55.8% and ceftazidime 34%). Only 338 *V. parahaemolyticus* isolates had 1-7 different plasmids and could be categorized into 27 patterns based on the number and pattern of plasmid present. Interestingly, there was no correlation between the number of plasmids and antibiotic resistant patterns seen in the isolates. The antibiotic resistance was mediated by both chromosomal and plasmid mediation among the resistant isolates. In summary, our results demonstrate that incidence of pathogenic *V. parahaemolyticus* in seafood in Selangor remains in relatively assuring levels, however the identification of antibiotic resistance among the isolates does rises a public health concern and warrants for continuous surveillance.

Keywords: Consumers; aquaculture; *Vibrio parahaemolyticus*; antibiotic resistance; Malaysia

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Introduction

The global public health is endlessly challenged by the threat of foodborne diseases and the latter restraints the socioeconomic development by affecting the healthcare system, country's economic, tourism and trade [(1,2)]. Since decades ago, there has been increasing number of foodborne diseases related with consumption of raw or undercooked foods in developed and developing countries [(3)]. Among the identified foodborne pathogens are *Salmonella* sp. [(4,5,6,7)], *Listeria* sp. [(8,9)] and *Vibrio* sp. [(10,11,12,13)] that are often associated with gastroenteritis cases

worldwide.

Vibrio parahaemolyticus – a member of the *Vibrionaceae* family is a Gram-negative, rod-shaped halophilic bacterium that naturally lives in aquatic environments [13,14]. *V. parahaemolyticus* is widely distributed in marine and estuarine environments thus causing gastrointestinal illnesses after being eaten raw or undercooked seafood [15,16]. During the 2016, the Centers for Disease Control and Prevention (CDC), United States reported that *V. parahaemolyticus* was acclaimed as the major foodborne bacterium compared to other *Vibrio* sp. This pathogen is accounted for nearly 34,664 foodborne cases annually in the United States [17,18].

In Malaysia, *V. parahaemolyticus* is naturally identified in the marine coastal region of Malaysia in all seasons and known to cause foodborne gastroenteritis [19]. In the early 1980s, a study revealed the incidence of *V. parahaemolyticus* in Malaysian shrimp processing industry. It is of interest to note that 21 different serotypes were isolated from Malaysian shrimp, with type 01:K38 and 01:K32 were predominates [20]. In addition, the EU countries has rejected the import of frozen black tiger shrimp from Malaysia due the presences of *V. parahaemolyticus* which subsequently affected the Malaysian economic [21]. The virulent *V. parahaemolyticus* carrying *tdh* and/or *trh* genes was also identified from the frozen shrimps in Malaysia, prompting a possible health risk for people consuming raw shrimp [22].

The food safety in Malaysia is further declining due to the rising cases of the detection of antibiotic resistant *V. parahaemolyticus* strains in seafood and environmental samples. There has been many reported cases of antibiotic resistant *V. parahaemolyticus* strains isolated from seafood namely shellfish, fish, and shrimps from Malaysia [10,11,19,23,24,25,26]. Antibiotics and other chemotherapeutic agents are often incorporated as feed additives or immersion baths in aquaculture farms to control bacterial infections [27,28,29]. Though antibiotics are effective in controlling bacterial infections, the misuse of antibiotics has caused the occurrence of multidrug resistant bacteria in the environments [25]. Henceforth, there is a need for appropriate management and control of the use of antibiotics in the aquaculture sectors.

The increase in bacterial resistance to many clinical antibiotics effects many country's healthcare and food production sectors [30]. In agreement with previous reports and the expected severity of infections, constant investigation on antimicrobial resistance of *V. parahaemolyticus* is needed for epidemiological purpose and guidance in healthcare treatment. For this reason, our study aimed to characterize antibiotic resistance of *V. parahaemolyticus* from shrimp and shellfish in Selangor, Malaysia.

Materials and Method

Bacterial Strains

V. parahaemolyticus isolates from previous study was used for the present study [10,11]. A total of 385 *V. parahaemolyticus* isolates were from shrimp and shellfish – red prawn (*Solenocera subnuda*), banana prawn (*Penaeus indicus*), mud crab (*Scylla serrate*), flower crab (*Portunus pelagicus*), carpet clam (*Paphia texile*), hard shell clam (*Meretrix meretrix*), and mud creeper (*Cerithidea obtuse*) was collected from wetmarket and supermarket in Selangor, Malaysia. All these isolates were confirmed *V. parahaemolyticus* by *toxR*-PCR assay and thermostable-related direct haemolysin (*trh*) gene was detected in the isolates [10,11].

Antibiotic Susceptibility Test (AST)

The antibiotic susceptibility of *V. parahaemolyticus* isolates was determined using Kirby-Bauer disc diffusion method [31]. Fourteen different types of antibiotics discs (Oxoid, UK) was tested: ampicillin (10µg), ampicillin/sulbactam (30µg), amikacin (30µg), cefotaxime (30µg), ceftazidime (30µg), chloramphenicol (30µg), gentamicin (30µg), imipenem (10µg), kanamycin (30µg), levofloxacin (5µg), nalidixic acid (30µg), oxytetracycline (30µg), suphamethox/trimethoprim (25µg), and tetracycline (30µg). *E. coli* ATCC 25922 with known sensitivity pattern was included as a positive control in each test.

V. parahaemolyticus isolates was grown in tryptic soy broth (TSB) (HiMedia, India) supplemented with 2% w/v sodium chloride (NaCl) (Vivantis, USA) at 37°C for 18 hours under constant agitation [13]. The bacteria cultures are lawn onto Mueller Hilton agar (HiMedia, India) with 2% w/v sodium chloride (NaCl) (Vivantis, USA), placed with antibiotic discs and incubated at 37°C for 18 hours. The zone of inhibition was measured and interpreted following the guidelines of Clinical and Laboratory Standards Institute (CLSI) M45-A2 [32]. The multiple antibiotic resistance (MAR) index was calculated based on the ratio isolate's resistance to the total number of tested antibiotics [33].

Plasmid Profiling

Plasmid profiling was carried out following the method adapted from previous study with slight modification [27]. *V. parahaemolyticus* cell were grown in tryptic soy broth (TSB) containing 2% w/v sodium chloride and incubated at 37°C in a shaker incubator (220rpm) for 18 hours. About 1.5 mL of the culture was transferred into a micro-centrifuge tube followed by centrifugation (10,000 rpm for 2 minutes at 4°C). The supernatant was removed by aspiration leaving the cell pellet as dry as possible. The pellet was resuspended in ice-cold 100ul alkaline lysis solution I (Glucose 50mM; Tris Cl 25mM; EDTA 10Mm) by vigorous vortexing followed by addition of freshly prepared 200uL alkaline lysis solution II (NaOH 2N; SDS 2% w/v). The contents were mixed by vortexing rapidly after which 150ul ice-cold solution III (Potassium acetate 5M: 60ml; Glacial acetic acid 11.5ml; dissolved in 28.5m sterile distilled water) was added to it. The tube was closed and gently vortexed for 10 seconds to disperse solution III through the viscous bacterial lysate.

Then the tubes were stored in ice for 5 minutes before being centrifuged at 12,000 rpm for 2 minutes at 4°C. An equal volume of phenol-chloroform (1:1, w/v) was added to the supernatant in a fresh tube, by vortexing. The contents in the micro-centrifuge tube were centrifuged

at 8,000 rpm for 3 minutes at 4°C and the supernatant was transferred into a fresh tube. This was repeated with chloroform: isoamyl-alcohol (24:1, v/v) for removing the phenol. The double stranded DNA was precipitated with 2 volumes of ethanol at room temperature, followed by vortexing before it was allowed to stand for 5 minutes at room temperature. The aliquot was centrifuged at 12,000 rpm for 12 minutes at 4°C and the supernatant was removed by gentle aspiration. The pellet of double stranded DNA was rinsed with ethanol (1ml, 70% v/v) at 4°C and centrifuged. The supernatant was removed leaving the pellet dry as possible. The pellet was air-dried before it was re-dissolved in 30ul ultrapure water. Electrophoresis was performed using 1% agarose gel.

Plasmid Curing

The antibiotic resistance mediation of *V. parahaemolyticus* isolate was determined by plasmid curing method using two different intercalating agent, acridine orange (AO) and ethidium bromide (EB) [10,34]. The isolates were revived in freshly prepared tryptic soy broth (TSB) supplemented with 0.2 mg/mL of respective curing agent and the tubes were incubated at 37°C for 18 hours under constant agitation. The treated culture was subjected to antibiotic susceptibility test as described in section 2.2 to re-examine the antibiotic resistance profiles. The phenotype results were compared with the antibiotic phenotype of non-treated isolate. The plasmid profiling as described in section 2.3 was performed with the treated culture in order to determine and compared the presences of plasmids before and after treatment.

Statistical Analysis

The data analysis was performed using IBM SPSS statistical analysis software version 20. Statistical analysis was performed to determine whether there is any significant difference in type of samples and MAR index of resistant *V. parahaemolyticus* isolates. A One-way analysis of variance (ANOVA) followed by suitable post-hoc test (Turkey) was used and $p < 0.05$ is considered as significant.

Results

Antibiotic Resistance of *V. parahaemolyticus* Strains

Fourteen antibiotics belonging to β-lactams, aminoglycosides, carbapenems, quinolones, tetracycline, sulphonamides, and chloramphenicol were used for the determination of antibiotic susceptibility of *V. parahaemolyticus* isolates. As shown in Table 1, a large number of isolates showed resistance to ampicillin (85%), amikacin (66.8%), and kanamycin (50.1%). A notable resistance pattern could be observed to the third generation cephalosporins (cefotaxime 55.8% and ceftazidime 34%). In contrast, high susceptibility rate was seen to imipenem (94%), chloramphenicol (92.5%), tetracycline (83.1%), ampicillin-sulbactam (81%), levofloxacin (76.1%), trimethoprim-sulfamethoxazole (75.8%), nalidixic acid (73.85), and gentamicin (70.6%). A high percentage (68%) of isolates have a significant MAR index more than 0.2. The value of MAR index ranged from 0.00 to 0.79, with the highest MAR index attributed from two isolates respectively (VP152 from supermarket banana prawn and SVP129 from supermarket carpet clam) exhibiting resistance profile towards 11/14 antibiotics tested.

Antibiotics	No. of resistant isolates (%)	No. of intermediate isolates (%)	No. of susceptible isolates (%)
Ampicillin (10ug)	327 (85)	29 (7.5)	29 (7.5)
Ampicillin-sulbactam (30ug)	41 (10.6)	32 (8.3)	312 (81)
Cefotaxime (30ug)	215 (55.8)	51 (13.2)	119 (30.9)
Ceftazidime (30ug)	131 (34)	98 (25.5)	156 (40.5)
Imipenem (10ug)	5 (1.3)	18 (4.7)	362 (94)
Amikacin (30ug)	257 (66.8)	90 (23.4)	38 (9.9)
Gentamicin (30ug)	28 (7.3)	85 (22.1)	272 (70.6)
Kanamycin (30ug)	193 (50.1)	161 (41.8)	31 (8.1)
Tetracycline (30ug)	57 (14.8)	8 (2.1)	320 (83.1)
Oxytetracycline (30ug)	67 (17.4)	108 (28.1)	210 (54.5)
Nalidixic acid (30ug)	38 (9.9)	63 (16.4)	284 (73.8)
Levofloxacin (5ug)	31 (8.1)	61 (15.8)	293 (76.1)
Trimethoprim-sulfamethoxazole (25ug)	18 (4.7)	75 (19.5)	292 (75.8)
Chloramphenicol (30ug)	22 (5.7)	7 (1.8)	356 (92.5)

Table 1: The percentage of antibiotic resistant *V. parahaemolyticus* isolates isolated from shrimp and shellfish samples.

This study revealed a high percentage of susceptibility towards imipenem, however it should be noted that five of the isolates (VP71, SVP90, VP114, VP145, and VP146) exhibited resistance to imipenem. Although the resistance to imipenem is only 1.3% of the total isolates, it still warrants a concern on the use of antibiotics as Carbapenems are among the beta-lactams that are the last line of antibiotic used for bacterial treatment [35]. These five isolates had MAR index of 0.21 to 0.64, and resistant to more than two different type of antibiotic tested. Imipenem resistance profiles was observed among isolates isolated from both shrimp and shellfish samples, demonstrating that the resistance occurred in different seafood samples regardless the habitat of marine organism. The VP114, VP145 and VP146 isolates was isolated from the banana prawn samples whereas VP71 was isolated from the red prawn and SVP90 was isolated from the flower crab sample.

Interestingly, the 32 *trh*-positive *V. parahaemolyticus* exhibited resistance to more than two different type of antibiotic tested (Table 2). Of the thirty-two isolates, 30 *trh*-positive isolates were seen resistant to ampicillin. Isolate SVP54 demonstrated resistance to six different antibiotics tested including ampicillin, amikacin, ceftazidime, cefotaxime, kanamycin, and levofloxacin. The 32 *trh*-positive isolates had MAR index of 0.21 to 0.64, with 62.5% (20/32) isolates are resistance to three and more different types of antibiotics tested. The presence of multi-resistant *trh*-positive isolates in the marine environment may hamper clinical treatment if one gets infected with these strains. This emphasises the need for frequent monitoring of seafoods.

Based on the One-way ANOVA analysis, there was a significant effect ($p < 0.05$) between type of samples and MAR index of *V. parahaemolyticus* isolates. In line with Tukey's Post Hoc analysis, there was a significant difference in the mean MAR index ($p < 0.05$) of *V. parahaemolyticus* isolates between red prawn and all the other type of seafood. There was a significant difference in the mean MAR index between banana prawn with red prawn, $p = 0.000$ ($p < 0.05$). The MAR index of swimming crab sample was significantly different with MAR index of red prawn ($p = 0.000$) and carpet clam ($p = 0.041$) ($p < 0.05$). There was no significant difference in the mean MAR index of *V. parahaemolyticus* isolates between hard shell clam and all the other type of samples, except for red prawn, $p = 0.000$ ($p < 0.05$). Figure 1 illustrates the comparison of mean MAR index of *V. parahaemolyticus* isolates from different type of seafood.

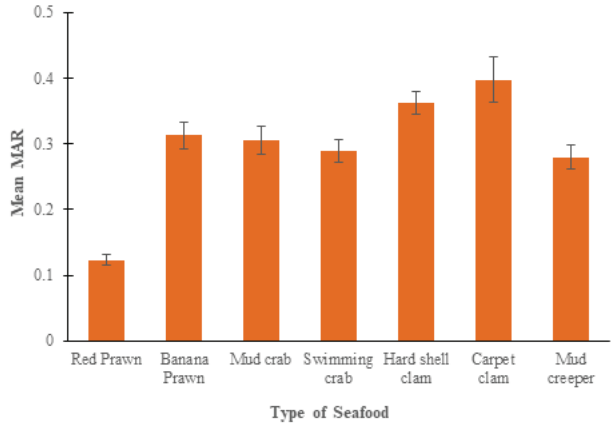

Figure 1: Comparison of the mean MAR index of *V. parahaemolyticus* isolates from different type of seafood. Each bar represents mean MAR index of isolates from type of seafood. The vertical lines associated with the bars represent two times the standard error of the mean.

Plasmid Profiles of *V. parahaemolyticus*

Three hundred and eighty-five *V. parahaemolyticus* isolates were analyzed for the presence of plasmids. Only 338 *V. parahaemolyticus* isolates have 1-7 different plasmids (Figure 2) and could be categorized into 27 patterns based on the number and pattern of plasmid present. The sizes of plasmids ranged from 1.2kb to above 10kb. As shown in Figure 2, from 27 plasmid profiles, the profile that forms the largest group was the plasmid profile 1.3 that consisted of 1 band above 10kb size plasmid. A total of 95 isolates (24.7%) have plasmid profile 1.3. Additionally, in this profile, 22 isolates were from shellfish samples and 73 isolates were from shrimp samples. The isolates grouped in this plasmid profile were identified to be resistant to at least one typed of the antibiotic tested. The isolate VP152 from supermarket banana prawn and isolate SVP129 from supermarket carpet clam which exhibited resistance profile towards 11/14 antibiotics tested respectively were grouped under plasmid profile 1.3. Isolate VP183 that was resistant towards 5/14 antibiotic tested (AK/AMP/C/OT/TE) and SVP61, a *trh*-positive isolate resistant towards 4/14 antibiotic tested (AMP/CTX/AK/CAZ) respectively harboured seven plasmids each. Overall, a total of 47/385 isolates (12%) did not express any plasmid profiles. The results demonstrated high discriminatory power of plasmid profiling conducted in this study.

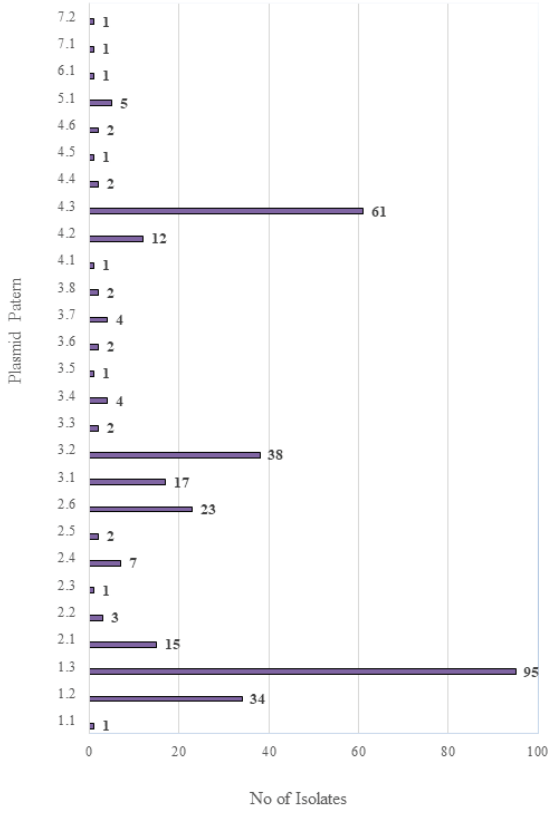

Figure 2: Bar Chart on plasmid profile of 385 *V. parahaemolyticus* isolates. The Y-axis represents different type of plasmid pattern while the X-axis represents the number of isolates that possess the particular pattern.

Table 2 shows an interesting relationship between the antibiotic resistance and plasmid profiles of the 32 *trh*-positive *V. parahaemolyticus* isolates. 21/32 *trh*-positive isolate contained 1-7 plasmids, where else another 11 isolates did not exhibit any plasmid profiles. All the *trh*-positive isolates were resistant to at least one type of antibiotic tested in study except isolate VP98 that was not resistant to any antibiotic and did not have any plasmid profile.

Isolates	Antibiogram	MAR Indes
SVP54	amp/ak/caz/ctx/k/lev	0.43
SVP55	amp/ak/caz/ctx/k	0.36
SVP56	amp/ak/caz/ctx/k	0.36
SVP70	amp/ak/caz/ctx/k	0.36
VP102	amp/ctx/ak/caz/k	0.36
VP103	amp/ctx/ak/caz/k	0.36
SVP61	amp/ak/caz/ctx	0.29
SVP66	amp/ak/caz/ctx	0.29
SVP69	amp/ak/caz/ctx	0.29
SVP72	amp/ak/caz/ctx	0.29
SVP75	amp/ak/ctx/k	0.29
VP90	amp/ctx/ak/caz	0.29
VP95	amp/ctx/ak/k	0.29
SVP73	ak/ctx/k	0.21
SVP64	amp/ak/ctx	0.21
SVP52	amp/ak/ctx	0.21
VP93	amp/ak/k	0.21
VP101	amp/ak/k	0.21
VP89	amp/ctx/ak	0.21
VP91	amp/ctx/ak	0.21
VP178	amp/ak	0.14
VP99	amp/ctx	0.14
VP175	amp/ctx	0.14
SVP60	AMP	0.07
VP92	amp	0.07
VP96	amp	0.07
VP97	amp	0.07
VP176	amp	0.07
VP177	amp	0.07
VP94	amp	0.07
VP100	amp	0.07
VP98		0.00

Table 2: Antibiotic resistant profile of *trh*-positive *V. parahaemolyticus* isolates

Ampicillin (AMP), Oxytetracycline (OT), Nalidixic acid (NA), Chloramphenicol (C), Cefotaxime (CTX), Sulfamethoxazole/Trimethoprim (SXT), Imipenem (IMP), Amikacin (AK), Ampicillin/Sulbactam (SAM), Levofloxacin (LEV), Ceftazidime (CAZ), Kanamycin (K), Gentamicin (CN), Tetracycline (TE).

Plasmid Curing

In this study, two different intercalating agents – acridine orange (AO) and ethidium bromide (EB) were used to determine the antibiotic resistance mediation. The plasmid curing revealed that both intercalating agents AO and EB produced same curing profiles of isolate and the results is demonstrated in Figure 3.

All 338 *V. parahaemolyticus* isolates that harbour 1-7 different plasmid ranging of size 1.2kb to above 10kb in size lost their plasmids upon being subjected to curing agents. In Figure 3, it could be observed that 327 *V. parahaemolyticus* isolates that were resistant towards ampicillin before plasmid curing showed the same phenotype resistance after plasmid curing. Similar resistance pattern could be observed in a group of 57 tetracycline resistant isolates. The plasmid curing results revealed that 51/57 isolates (89%) were still resistant towards tetracycline. This suggests that the resistance phenotype to ampicillin and tetracycline expressed by the isolates could be chromosomally mediated. All the ampicillin/sulbactam resistant strains lost their plasmid after the curing assay and subsequently were susceptible to ampicillin/sulbactam suggesting it was plasmid mediated resistance. The antibiotic resistant patterns of OT/C/CTX/SXT/AK/CAZ/K/CN presented after plasmid curing had lower number of resistant isolates towards respective antibiotic. These results demonstrate that the phenotype resistance observed could be both plasmid and chromosomal mediated.

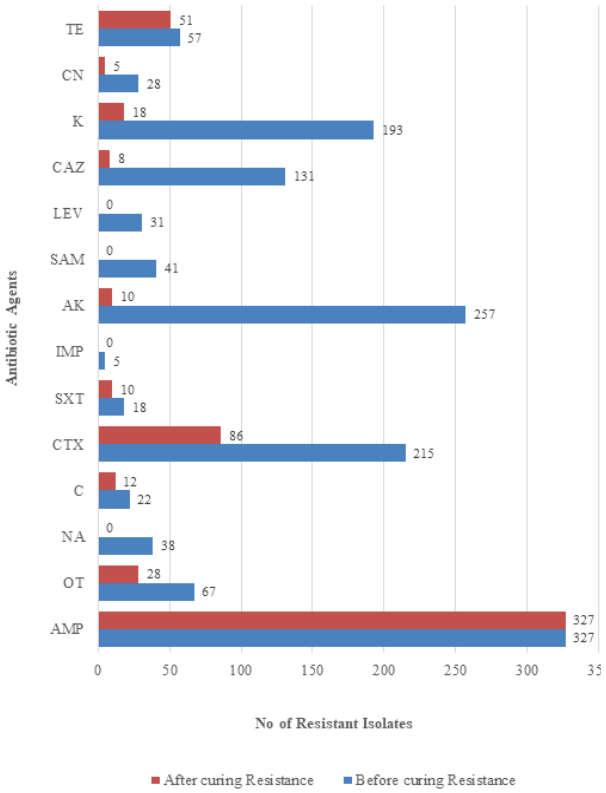

Figure 3: Bar Chart on antibiotic resistance profile of *V. parahaemolyticus* before and after plasmid curing. The Y-axis represents different type of antibiotic agents while the X-axis represents the number of resistant isolates towards the antibiotic agents. Ampicillin (AMP), Oxytetracycline (OT), Nalidixic acid (NA), Chloramphenicol (C), Cefotaxime (CTX), Sulfamethoxazole/Trimethoprim (SXT), Imipenem (IMP), Amikacin (AK), Ampicillin/Sulbactam (SAM), Levofloxacin (LEV), Ceftazidime (CAZ), Kanamycin (K), Gentamicin (CN), Tetracycline (TE).

With reference to the 32 *trh*-positive *V. parahaemolyti-*

cus isolates (Table 2), the antibiotic resistance profile of the 21 plasmid containing isolates changed after curing while the remaining 10 were unchanged. All 20/21 were ampicillin resistant initially, and after curing, the isolates (SVP61, SVP54, SVP75, SVP69, SVP72, VP89, VP90, VP91, VP92, VP93, VP94, VP95, VP99, VP101, VP102, VP102, VP103, VP1175, VP176, VP177, VP178) remained resistant to ampicillin and cefotaxime, and became susceptible to the other antibiotics tested. One isolate (SVP73) became susceptible to all antibiotic resistant after plasmid curing, suggesting the resistance phenotype observed was plasmid mediated. This suggests that while antibiotic resistance is mediated by both plasmid and chromosomes in pathogenic *V. parahaemolyticus* isolates, in plasmid containing strains aside from ampicillin and cefotaxime resistance, most of the remaining resistance phenotypes are plasmid mediated.

SVP129 isolate contained one plasmid profile with size more than 10kb and expressed antibiotics resistance towards 11/14 antibiotics tested. After plasmid curing, SVP129 isolate lost its plasmid and changed its antibiotic resistance phenotype. SVP129 isolate remained resistant to ampicillin, oxytetracycline, chloramphenicol, tetracycline and sulfamethoxazole/trimethoprim; intermediate resistance to amikacin, ceftazidime, cefotaxime and kanamycin; and susceptible to gentamycin and ampicillin/sulbactam after plasmid curing assay.

Discussion

In view of many reported cases of antibiotic resistance of *V. parahaemolyticus* from aquaculture, healthcare personals and members of the public should be caution in the application of antibiotics in healthcare sectors and aquaculture sectors. The rising number of antibiotic resistance as well as resistant genes within the *V. parahaemolyticus* population does causes a global health issue [36-44]. Hence, continuous monitoring is required to review the antibiotic resistance patterns and followed by controlling the use of antibiotics in the environments.

The study's susceptibility test placed the 1st generation antibiotic – ampicillin at the top of the *V. parahaemolyticus* resistance scope (85%) (Table 1). This result is in close agreement with previous reports from India, Indonesia, Korea and Malaysia that reported prevalent of ampicillin resistant *V. parahaemolyticus* strains isolated from seafood samples [10,19,27,45-50]. The 1st generation antibiotics including ampicillin has a very low efficacy in treatment of infections due to the misuse of these antibiotics in aquaculture and agriculture which in turn led to a low susceptibility rate [25]. These findings signify that ampicillin may longer be an effective antibiotic to treat *Vibrio* sp. infections. The occurrence of high ampicillin resistance rate in the environment is still of great concern since the resistance phenotype seen could be chromosomally mediated in the bacteria thus require proper management method to control the resistance phenotype [13].

Interestingly, multi-resistance profile was observed among the 32 *trh*-positive *V. parahaemolyticus*. These pathogenic isolates were seen resistant to aminoglycosides, 3rd generation cephalosporins, and quinolone. The *V. parahaemolyticus* isolates exhibited high resistance rate towards the 3rd generation cephalosporins – cefotaxime (55.8%) and ceftazidime (34%) in this study. These findings are in line agreement with a study from Terengganu, Malaysia, who reported ceftazidime and cefuroxime resistant *V. parahaemolyticus* isolates from shellfish [51]. In the neighboring country, Korea, another similar study reported high percentage (70%-80%) of *V. parahaemolyticus* isolates from Korean seafood to be resistant to the 3rd generation cephalosporin, cefotaxime and ceftazidime [52]. In contrast, a study from the US reported low percentage of cefotaxime resistant *V. parahaemolyticus* isolates isolated from food [53]. The inconsistencies in *V. parahaemolyticus* resistance rate to 3rd generation cephalosporin may be due to difference in sample, geographical variations, or difference in methodology test applied.

It is reassuring to note that the isolates in this study are still susceptible to some antibiotics tested including imipenem (94%) (Table 1). Nevertheless, there were five isolates (SVP90, VP71, VP114, VP145, VP146) exhibited resistance towards imipenem. The detection of imipenem resistant isolates raises concern as carbapenems are the most potent **β**-lactam antibiotic and is usually administrated in treatment of any serious bacterial infections [35]. The results are in agreement with previous reports on the isolation of carbapenem-resistant *Vibrio* sp. from environmental samples. Walsh and colleagues reported carbapenem resistant *V. cholerae* isolated from drinking water and seepage in New Delhi, India and further analysis revealed that the carbapenem gene bla_{NDM-1} was found in the chromosome of *V. cholerae* isolate [54]. The occurrence of carbapenem resistance was also detected in a *V. cholerae* 01 El Tor Ogawa strain isolated from faecal specimen of a 2-year-old child in Puducherry, India. Another study reported an increasing trend of carbapenem resistance among *V. cholerae* 01 or 0139 isolates between 1986 to 2012 in southwest China [55]. Recently, Bier and colleagues reported the isolation of four carbapenem resistant *V. cholerae* from different locations of the German coast line. These four isolates were not only resistant to carbapenem but also exhibited resistance to cefoxitin, aztreonam, and aminopenicillin [56]. In addition, there have been reports on the emergence and spread of carbapenem-resistant *Enterobacteriaceae* (CRE) in the United States (US) [57,58]. Any infections with carbapenem resistant bacteria may cause higher mortality rates compared to those infections caused by carbapenem-susceptible bacteria. The wide incidence of carbapenem resistant *Vibrio* sp. is an important emerging threat to public health, thus requires proper management action to limit the spread of this organism.

In this study there was no significant difference between the sampling location and MAR index of *V. parahaemolyticus* isolates. This result demonstrates that the isolates isolated from wetmarket and supermarkets are exposure to antibiotics. Our results came to an agreement with many studies that reported high percentage of *V. parahaemolyticus* isolated from seafood are resistant to more than one antibiotic tested [28,29,48,59,60]. According to the One-way ANOVA analysis results, there was a significant difference between the groups of sampling location

on the MAR index of *V. parahaemolyticus* isolates ($p < 0.05$). The isolates from the supermarket sampling sites had a higher mean MAR index compared to the isolates from wetmarket sampling sites. This situation could be attributed by the geographical difference in seafood samples that been sold in supermarket, thus causing a MAR resistance profile. In addition, it could be suggested that seafood samples may have originated from similar environmental conditions in terms of antibiotic exposure or cross contamination may have occurred during the post-harvest, resulting in the isolates to have similar MAR index.

When compared the antibiotic resistance patterns and plasmid profiles, there was no correlation observed. Even within the isolates with same resistance profiles, the plasmid profiles were different and a few isolates even did not exhibit any plasmids, which was similar to findings by Lajnef and colleagues [61]. Hence, it could be concluded that the antibiotic resistance is not been influenced by the number of plasmids acquired by the isolates. The exposure of antibiotic in environment causes the bacteria to display a multidrug resistant characteristic. In some strains, the resistance observed could be plasmid mediated, and in some are chromosomally coded. Further research could be done to confirm the origin of antibiotic resistance among the isolates.

In this study, two different plasmid curing agent, acridine orange (AO) and ethidium bromide (EB), both are intercalating agents. Two different intercalating agents were used because to observed the efficacy of each agent. Intercalating agents such as AO and EB have been successfully used many studies of curing bacterial plasmids [31,37,62-66]. The modes of action of intercalating agents are through preferential inhibition of plasmid replication. Both the intercalating agents yield the similar curing profiles for each isolate (Figure 4.8).

The present results are closely in agreement with other studies that reported *Vibrio* sp. isolates lost their plasmids when treated with concentration of 0.2mg/ml acridine orange (AO) and the isolates demonstrated changes in their resistance profile [64-67]. A Brazilian study reported AO agent was successfully used to cure multi-resistant *Vibrio* isolates from marine shrimp and concluded the ampicillin resistance strains in study are plasmid mediated [37]. In contrast, another study reported their isolates resistance was chromosomal mediated after AO curing agent treatment [66]. Likewise, another study reported the alteration in antibiotic resistance patterns and loss of plasmid among *Vibrio* sp. isolates when treated with 0.3mg/mL EB. In that study 79% of the *Vibrio* sp. isolates loss their plasmid profiles but showed phenotype resistance pattern to amoxicillin, ampicillin, furazolidone and tetracycline after curing assay, which indicate the resistance may be chromosomally borne [28].

Conclusion

In conclusion, the current study provides an overview on the seafood contamination levels in Selangor, Malaysia and the distribution of *V. parahaemolyticus* in shrimp and shellfish samples. The shrimp and shellfish samples analyzed were contaminated with *V. parahaemolyticus* regardless their sampling locations. There was no correlation observed between the antibiotic resistance and plasmid profiles. Yet, the antibiotic resistance mediation was studied via the plasmid curing assay. In some isolates, the resistance was plasmid mediated, while others were chromosomally borne resistance. The information derived from this curing assay is useful for public health personnel to understand better on the antibiotic resistance of *V. parahaemolyticus* in shrimp and shellfish from Selangor, Malaysia. The plasmid curing assay is fast, cost saving, provides fundamental knowledge, and may influence effective antibiotic management policies in the aquaculture industry. With this knowledge, the aquaculture farmers may alternate the antibiotics in their aquaculture fields from time to time in order to allow withdrawal of antibiotic resistance among the bacteria [34]. In summary, the antibiotic resistance presented by *V. parahaemolyticus* isolates could be due to the excessive use of antibiotic in aquaculture to control bacterial infection and huge production loses [68,69]. In addition, antimicrobial resistance is also caused by exposure of antibiotics via agriculture runoff, wastewater treatment plants, and thru mobile genetic elements or horizontal gene transfers among bacteria [70]. There have been prevalent cases of multiple resistance reported among environmental pathogens such as *Salmonella* sp. [71,72], *V. vulnificus* [73], *Listeria monocytogenes, Escherichia coli* and *V. parahaemolyticus* [74]. Hence, the present results would provide a baseline information on the severity of resistance among *V. parahaemolyticus* in shrimp and shellfish in Selangor Malaysia, then may allow management personal to overcome this problem with proper management strategies.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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