Prevalence, antimicrobial resistance profiles and virulence genes of *Vibrio* spp. isolated from shrimp retails in Ho Chi Minh City (Vietnam)

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ABSTRACT

This study was conducted to determine the diversity of pathogenic *Vibrio* species, the antimicrobial resistance profile and the presence of virulence genes linked to food-borne pathogens of *Vibrio* spp. isolated from shrimp samples in Ho Chi Minh City, Vietnam. A total of 40 raw shrimp batches were collected from retails markets (supermarket and street). All 133 test strains were isolated from 40 shrimp samples. V. parahaemolyticus was the most common species (87.5%), followed by *V. navarrensis* (60%), *V. alginolyticus* (52.5%), *V. cholerae* non-O1 (37.5%), *V. vulnificus* (22.5%), and *V. fluvialis* (10%). *Vibrio* spp. isolates were susceptible to 12 antimicrobial agents. The prevalence of ampicillin resistance was highest (82.7%), followed by cotrimoxazole (18.8%) and 3rd generation cephalosporins (16.5% cefotaxime and 8.3% ceftazidime). Extended-spectrum β lactamase (ESBL) activity was detected in 28.1% *V. parahaemolyticus* isolates. None of *tdh* or *trh* virulence genes were detected. The results of this study indicated the presentation of *Vibrio* species in shrimp samples purchased in Ho Chi Minh City. Therefore, our results could be of great potential for the identification of *Vibrio* infection in shrimp samples taken from different regions to improve food quality and safety.

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1. Introduction

Among several aquatic species, shrimp farming grew quickly in Vietnam, making the country the third greatest shrimp exporter globally (Giang, 2017). The diseases were caused by pathogens such as bacteria, fungi, parasites, and viruses decreased significantly the shrimp production. Bacteria particularly *Vibrio* species, hit global shrimp industry in southern and southeastern Asia (Otta et al., 2001). *Vibrio cholerae* caused cholera, a severe diarrheal disease that could be life-threatening if untreated. It could spread through contaninated water and person to person contact. Non-cholera *Vibrio* spp. (for example, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, and *Vibrio vulnificus*) caused Vibriosis. Thermostable direct hemolysin (*tdh*) and tdh-related hemolysin (*trh*) are considered major virulence of *Vibrio parahaemolyticus*. Clinical strain commonly contain either these genes and the presence of these genes is associated with pathogenicity of the strain in human (Baker-Austin et al., 2018). In the late 1990s', *V. parahaemolyticus* was implicated in a large outbreak of enteric disease in central Vietnam, with 523 cases reported (Chowdhury et al., 2004). The antibiotics and other drugs were used for growth promotion and disease prevention and treatment in shrimp culture. The usage of antibiotics in

shrimp culture have increased significantly over the last ten years in Viet Nam (Thuy et al., 2011). The misuse of antibiotics led to the proliferation of antibiotic resistance repoted in *Vibrio* strains (Elmahdi et al., 2016).

The aims of this study were to investigate the prevalence of *Vibrio* spp. in shrimps as well as analyzing the antimicrobial susceptibility profile including the presence of ESBL and their virulence genes.

2. Materials and Methods

2.1. Sample collection

Batches of shrimps (250 - 300 g each) were purchased from 40 different retail sites in 10 districts of Ho Chi Minh City (Vietnam) from March to June 2018. Shrimps which collected in sterile plastic bags (either in live/dead/unfrozen condition) to avoid cross contamination and were transported to the laboratory within 2 h in an ice-containing box. A total of five representative specimens per batch were weighted by using precision scales. three street markets and one supermarket were selected each district. From each retail site, shrimp was purchased in two forms: live or dead (chilled, not frozen). Each batch was collected the information of shrimp species. The heads, legs, and exoskeletons were separated aseptically from the muscle tissue by using a pair of sterile scissors and were subsequently pooled (shell mix). The shell mixes were used to investigate *Vibrio* spp.

2.2. Isolation of *Vibrio* spp. from the samples

Twenty-five gram of shrimp shell mixes from each sample was enriched in 225 mL of alkaline saline peptone water (ASPW) with 2% NaCl (pH 8.6) at 41.5^0C for 24 h. After enrichment, a loop of the inoculum was streaked onto Thiosulfate citrate bile and sucrose agar (TCBS) at 37^0C for 24 h (Figure 1). The colonies showed typical phenotypic characteristics of *Vibrio* spp. were identified by Maldi-tof (Bruker, Germany).

2.3. Antimicrobial susceptibility testing

The antimicrobial susceptibility of *Vibrio* spp. isolates were determined by using the disk diffusion method. The isolates were classified as resistant, intermediate and sensitive according to the guidelines of the Clinical and Laboratory Standard Institute (M45-A2 2006, CLSI, 2016). Multidrug resistance (MDR) was defined as fully resistant to at least three antimicrobial classes. *Escherichia coli* ATCC 25922 and *Klebsiella pneumonia* ATCC 700603 strain were used for quality control purposes. (Elmahdi et al., 2016).

Briefly, 2 - 3 colonies from MEA were transferred to sterile saline solution (0.9%) by stir loop until the saline solution achieved a turbidity equivalent to a 0.5 McFarland standard. Then, the solution was spread on Mueller Hinton agar by using sterilized swabs. A disk diffusion test was used for susceptibility testing with 12 antimicrobials representative of eight classes of antimicrobials (Oxoid, UK). The full list of antimicrobials investigated is displayed in Tables 1. The plates were inverted and incubated at 37^0C for 18 to 24 h. The level of antimicrobial resistance was measured based on comparing the diameter of the inhibition zone on MHA with the standard of CLSI (CLSI, 2016).

The potential production of extended-spectrum β-lactamase (ESBL) was detected by a double disk synergy method using cefotaxime (10 μg), ceftazidime (30 μg) was placed 2 – 3 cm away from a clavulanate acid disk (Oxoid, Hampshire, England). Antimicrobial susceptibility testing results were presented according to the WHO list of antimicrobials ranked by their importance for human health. A clear extension of the edge of the inhibition zone of third generation cephalosporins towards the disk containing clavulanate was interpreted as positive for ESBL production.

ESBL production was confirmated by resistance to a third generation cephalosporin, which was performed by the combination disk diffusion method with cefotaxime and ceftazidime disks alone and in combination with clavulanate (CLSI, 2016).

2.4. Investigation of virulence gene of *Vibrio* spp. by PCR

PCR amplifications was done by the use of *tdh* and *trh* specific primers for detection of pathogenic isolates, as previously described by (Tada et al., 1992). They are genes encoding the thermostable direct hemolysin (*tdh*) and the thermostable direct hemolysin-related hemolysin (*trh*), both described as major virulence of the

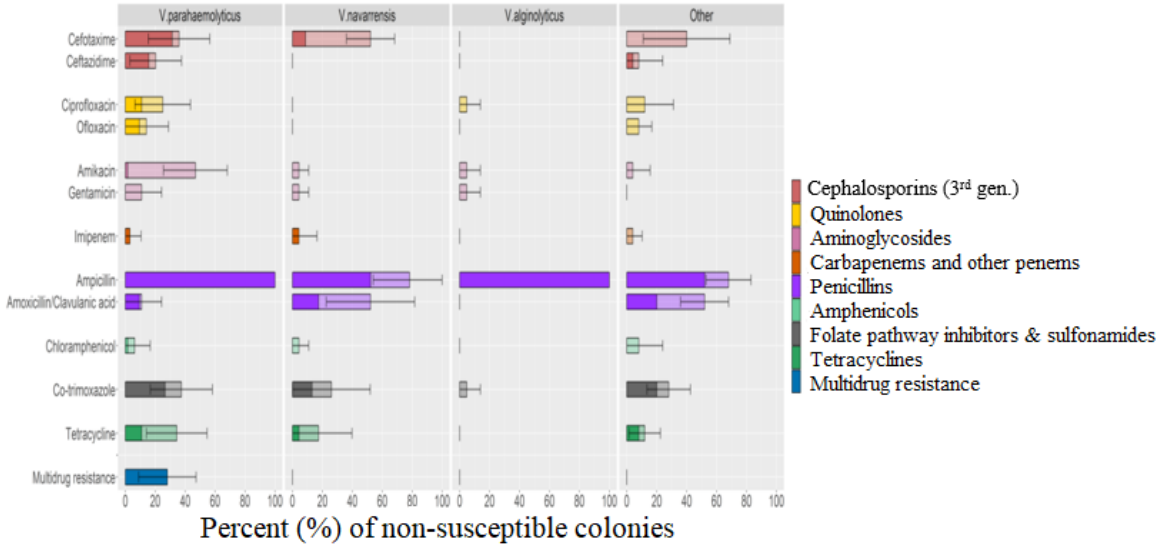

Figure 1. Phenotypic resistance amongst *Vibrio* spp. isolates by the group. The percent of isolates showing intermediate resistance was indicated by pale bars; dark bars indicate the percent of isolates with full resistance.

Table 1. Occurrence of Vibrio spp. in shrimp samples in Ho Chi Minh City, Vietnam

Variable	No. sample	No. of Vibrio species positive samples (%)					
		V.paraamlticus	*V. navarrensis*	*V. alginolgticus*	*V. cholerae non-O1*	*V. vulnificus*	*V. fluvialis*
Type of retail site							
Supermarket	10	9 (90.0%)	7 (70.0%)	4 (40%)	6 (60.0%)	2 (20.0%)	0 (0%)
Street market	30	26 (86.7%)	17 (56.7%)	17 (56.7%)	9 (30.0%)	7 (23.3%)	4 (13.3%)
Shrimp species							
White leg shrimp	30	26 (86.7%)	15 (50.0%)	16 (53.3%)	12 (40.0%)	7 (23.3%)	4 (13.3%)
Giant tiger shrimp	5	4 (80.0%)	5 (100%)	2 (40.0%)	2 (40.0%)	1 (20.0%)	0 (0%)
Other species	5	5 (100%)	4 (80%)	3 (60%)	1 (20%)	1 (20%)	0 (0%)
Condition							
Alive	17	16 (94.1%)	8 (47.1%)	11 (64.7%)	4 (23.5%)	5 (29.4%)	4 (23.5%)
Dead	23	19 (82.6%)	16 (69.6%)	10 (43.5%)	11 (47.8%)	4 (17.4%)	0 (0%)
Total	40	35 (87.5%)	24 (60.0%)	21 (52.5%)	15 (37.5%)	9 (22.5%)	4 (10.0%)

Table 2. Antimicrobial susceptibility of *Vibrio* spp. isolates

Class and antimicrobial	No. isolates and percent resistant (%)						
	V.paraamlticus (n = 64)	*V. navarrensis* (n = 23)	*V. alginolyticus* (n = 21)	*V. cholerae non-O1* (n = 15)	*V. vulnificus* (n = 6)	*V. fluvialis* (n = 4)	Total (n = 133)
Cephalosporins (3^{rd} gen.)	20 (31.3)	2 (8.7)	-	1 (6.7)	-	-	23 (17.3)
Cefotaxime	20 (31.3)	2 (8.7)	-	-	-	-	22 (16.5)
Ceftazidime	10 (15.6)	-	-	1 (6.7)	-	-	11 (8.3)
Quinolones	7 (10.9)	-	-	-	-	-	7 (5.3)
Ciprofloxacin	7 (10.9)	-	-	-	-	-	7 (5.3)
Ofloxacin	6 (9.4)	-	-	-	-	-	6 (4.5)
Aminoglycosides	1 (1.6)	-	-	-	-	-	1 (0.8)
Amikacin	1 (1.6)	-	-	-	-	-	1 (0.8)
Gentamicin	-	-	-	-	-	-	-
Carbapenems							
Imipenem	2 (3.1)	1 (4.3)	-	-	-	-	3 (2.3)
Penicillins							
Ampicillin	64 (100)	12 (52.2)	21 (100)	8 (53.3)	2 (33.3)	3 (75)	110 (82.7)
Amoxicillin-clavulanic acid	6 (9.4)	4 (17.4)	-	5 (33.3)	-	-	15 (11.3)
Amphenicols							
Chloramphenicol	1 (1.6)	-	-	-	0 (0)	-	1 (0.8)
Folate pathway inhibitors							
Trimethoprim/ sulfamethoxazole	17 (26.6)	3 (13)	-	3 (20)	2 (33.3)	-	25 (18.8)
Tetracyclines							
Tetracycline	7 (10.9)	1 (4.3)	-	1 (6.7)	1 (6.7)	-	10 (7.5)
ESBL	18 (28.1)	-	-	-	-	-	18 (13.5)
MDR	18 (28.1)	-	-	-	-	-	18 (13.5)

emergent human pathogen *Vibrio parahaemolyticus*, and other *Vibrio* spp. (Shirai et al., 1990; Terai et al., 1991). The *tdh* and *trh* genes were used to amplified which derived from patients showing typical symptoms of *Vibrio* infection.

DNA extraction process: Strains were subcultured to obtain individual colonies on MEA at 37^0C for 24 h. A single colony was suspended in 500 μL of nuclease-free water. DNA was extracted by heating at 95^0C for 10 min and centrifuged at 14000 rpm. A clear supernatant was used as the DNA template in a PCR.

PCR thermal cycling consisted of a 96^0C hold for 5 min for the initial denaturation, followed by 25 cycles of amplification, with each cycle consisting of denaturation at 94^0C for 1 min and an annealing step at 55^0C for 1,5 min, and an extension at 72^0C for 1,5 min. After the final extension at 72^0C for 7 min, the final hold at 12^0C to preserve all PCR samples.

A negative control containing nuclease-free H_2O. For positive controls, DNA were cloned and

confirmed by sequencing were used, which possessing *tdh* and *trh* genes.

The PCR products were electrophoresed in a 1.0% agarose gel, stained by ethidium-bromide at 160 V for 30 min and photographed. Positive reactions were identified by detecting a 250 bp and 251 bp specific band visualized on agarose gels under ultraviolet light.

2.5. Data analyses

The prevalence of *Vibrio* strains was evaluated in terms of percentage occurrences, in which the positive samples were compared to the total taken samples. The antimicrobial resistance of a group of isolates was calculated as the percentage of isolates among the group that was resistant to a single antimicrobial or a number of antimicrobials. Chi-square tests (χ^2 test) or Fisher's exact tests were used to compare these proportions using online statistical tools (Minitab Software). Statistically significant difference was defined if the value of $P < 0.05$.

3. Results

3.1. Prevalence of *Vibrio* spp. from the samples

The 40 batches included five species of shrimp: white leg shrimp (*Litopenaeus vannamei*) (30), giant tiger shrimp (*Penaeus monodon*) (5), banana shrimp(*Penaeus merguiensis*) (3), greasy-back shrimp (*Metapenaeus ensis*) (1), and giant prawn (*Macrobrachium rosenbergii*).

All (100%) samples were positive for *Vibrio* species. Among six *Vibrio* species, the most common species was *V. parahaemolyticus* (87.5%), followed by *V. navarrensis* (60%), *V. alginolyticus* (52.5%), *V. cholerae* non-O1 (37.5%), *V. vulnificus* (22.5%) and *V. fluvialis* (10%). The positive samples for *Vibrio* species results are shown in Table 1.

Statistical analysis was showed there was no significant difference among the sampling sites for the *Vibrio* spp. positive isolates. In addition, *V. navarrensis* prevalence was isolated more in giant tiger shrimp than in white leg shrimp ($P = 0.036$). *V. fluvialis* was also found more in shrimp that bought alive ($P = 0.014$).

3.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing against 12 antibiotic drugs was analyzed in 133 of *Vibrio* spp. isolates and the results are shown in Figure 1 and Table 1. The highest prevalence of resistance was ampicillin (82.7%), followed by trimethoprim sulfamethoxazole (18.8%) and 3rd generation cephalosporins (16.5% cefotaxime and 8.3% ceftazidime). All *V. parahaemolyticus* and *V. alginolyticus* isolates were resistant to ampicillin (100%). In total, the prevalence of resistance against amoxicillin-clavulanic,carbapenems, aminoglycosides, tetracyclines, quinolones and amphenicols was < 11.3% (Table 2).

ESBL was detected in 18/64 (28.1%) *V. parahaemolyticus* strains that were resistant to third- generation cephalosporins (31.3%). producer. The multidrug resistance to more than 3 of antibiotic classes was found in 13.5%. All non- *V. parahaemolyticus* isolates were negative for ESBL (18/133) *Vibrio* spp. isolates including the atypical *V. parahaemolyticus* isolates and a total of 15/18 (83.3%) ESBL positive *V.parahaemolyticus* strains were positive *V. parahaemolyticus* strains were positive for MDR.

3.3. Virulence gene of *Vibrio* spp. in shrimp samples

None of the virulence genes (*tdh* and *tdr*) were presented in 133 *Vibrio* spp. isolates. The representative gel photos for the PCR targeting trh and tdh gene were shown in Figure 2 and Figure 3, respectively.

4. Discussion

4.1. Prevalence of *Vibrio* spp. from the sample

Our study demonstrated that *Vibrio* spp. was dominent in shrimp samples sold in some retails in Ho Chi Minh City, Vietnam (100%). The occurrence of *Vibrio* species in tropical shrimp culture environments such as Vietnam might be expected because the areas with high temperate are an optimal condition for their growth and the infections are directly linked to this pathogen cannot be avoided in shrimp cultures because they are part of the natural microflora of coastal and estuarine environments (Koralage et al., 2012). In a previous study, similar results were ob-

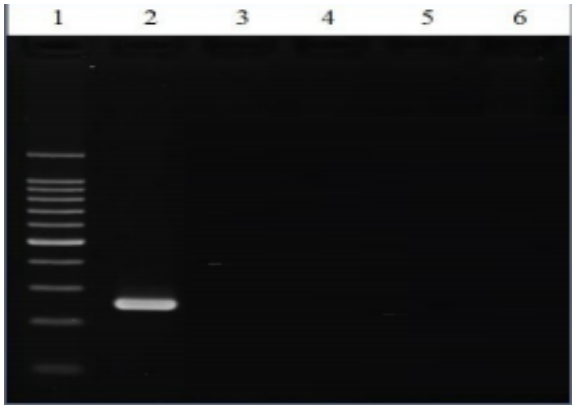

Figure 2. *tdh* gene in *V. parahaemolyticus* with size of 251 bp. Lane 1, 100bp DNA marker; lane 2, positive control; lane 3, negative control; 4-6 *V. parahaemolyticus* isolates.

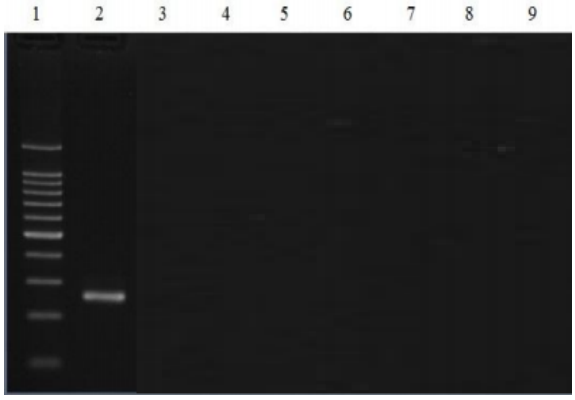

Figure 3. *trh* gene in *V. parahaemolyticus* with size of 250 bp. Lanes 1 and 10, 100bp DNA marker; lane 2, positive control; lane 3, negative control; 4-9 *V. parahaemolyticus* isolates.

tained in northern Vietnam (99.5%) (Tra et al., 2016) and Sri Lanka (98.1%) (Koralage et al., 2012). *V. parahaemolyticus* was the most prevalent species (87.5% samples). The predominance of *V. parahaemolyticus* in our study is similar to the study of retail shrimps in Ha Noi, Vietnam (96.5%) (Tra et al., 2016). However, *V. alginolyticus* was the predominant *Vibrio* species in another study (Sperling et al., 2015). In agreement with our study, *V. vulnificus* has been identified from shrimps in various countries at low prevalence (Gopal et al., 2005; Sperling et al., 2015). *V. parahaemolyticus* plays an important role because it causes diseases and mortality to the shrimp as primary and secondary pathogens. This strain found in previous study was recorded as a primary pathogen to White Spot Disease because population of the bacterial species increases with the onset of this viral disease. In addition, the result from other Antimicrobial susceptibility testing against 12 antibiotic drugs was analyzed in 133 of *Vibrio* spp. isolates and the results are shown in Figure 1 and Table 2. The highest prevalence of resistance was ampicillin (82.7%), followed by trimethoprim sulfamethoxazole (18.8%) and 3rd generation cephalosporins (16.5% cefotaxime and 8.3% ceftazidime). All *V. parahaemolyticus* and *V. alginolyticus* isolates were resistant to ampicillin (100%). In total, the prevalence of resistance against amoxicillin-clavulanic,carbapenems, study confirmed the potential of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* non-O1 as a major foodborne pathogens (Baker-Austin et al., 2017). The high prevalence of *V. parahaemolyticus* in shrimps is considered as potentially hazardous, regarding the probability of pathogenicity among the contaminant strains.

White leg shrimp is one of the most dominant farmed shrimp species in the world because of its fast growth and good toleration rate at high stock densities in different salinity levels (Cornejo-Granados et al., 2017). The white leg shrimps occupy high demand among the most consumed other shrimp in Vietnam markets.

The high prevalence of *V. fluvialis* was found more in shrimp that bought alive that indicated inadequate control in storage temperature which could be the condition in a proliferation of the pathogens. This reflected the scenario of retail outlets. As the cells of this pathogen are very sensitive to freezing and it can grow in the presence or absence of oxygen (Joseph et al., 1982).

4.2. Antimicrobial susceptibility testing

A total of 82.7% of *Vibrio* spp. isolates were resistant to ampicillin including 100% for *V. parahaemolyticus* and *V. alginolyticus*. High antimicrobial resistance to ampicillin and cephalothin were also reported (Lou et al., 2016; Rocha et al., 2016). These results are in agreement with those of other studies that indicated high resistance among *V. parahaemolyticus* isolates from shrimps, especially to ampicillin in northern Vietnam (87.2%) (Tra et al., 2016) and India (100%) (Vaseeharan et al., 2005).

Beta-lactam antibiotics are one of the main

groups used against Gram-negative and Gram-positive bacteria and account for 60% of the antibiotics used worldwide for the treatment of infectious diseases (Livermore & Woodford, 2006). The widespread use of ampicillin and cephalothin in aquaculture resulted in a reduction in the efficacy of treatment (Rocha et al., 2016). The result showed that the high percentage of ampicillin resistance was found in *V. parahaemolyticus* suggests that ampicillin cannot effectively treat infections caused by this organism. Another study reporting that *V. parahaemolyticus* isolated from shrimps in Hong Kong was positive to ESBL (Wong et al., 2012; Liu et al., 2013). The multidrug resistance to more than 3 of antibiotic classes was found in 13.5% (18/133) *Vibrio* spp. isolates including the atypical *V. parahaemolyticus* isolates and a total of 15/18 (83.3%) ESBL-positive *V. parahaemolyticus* strains were positive for MDR. According to results in Brazil, 50% of *V. parahaemolyticus* isolates presented multiple antibiotic resistance (de Melo et al., 2011). We found a high prevalence of MDR among *Vibrio* spp., with a particularly *V. parahaemolyticus* are probably a reservoir of these important resistance genes genes (Wong et al., 2012).

The misuse of antibiotics can increase resistance strains (Elmahdi et al., 2016). *Vibrio* spp. are considered as a genus of human pathogens, so antimicrobial resistance in aquatic environment threaten to human health. A rapid and accurate diagnostic method is necessary to detect the antimicrobial susceptibility of different strains. Management practices should be taken to reduce antimicrobial usage.

4.3. Virulence gene of *Vibrio* spp. in shrimp samples

No two major virulence genes positive *V. parahaemolyticus* strains were detected. Previous research showed the prevalence of *tdh* and *trh* genes in environmental, non- clinical and seafood-related strains was zero or very low (1 to 3%) (Gopal et al., 2005). In a study on 70 shrimp samples from Iran, only 2 (2.8%) *tdh* and 1 (1.4%) *trh* positive strains were identified (Asgarpoor et al., 2018). A total of 385 seafood samples in the Mekong Delta of Vietnam including tdh gene positive *V parahaemolyticus* strains were 22 (5.7%) samples and trh gene positive *V. parahaemolyticus* strains were 5 (1.3%) samples (Tran et al., 2018). However, there was no evidence of these genes among isolates investigated in northern Vietnam (Tra, et al., 2016), or Sri Lanka (Koralage et al., 2012). In addition, the results of other studies indicate that even nontoxigenic *V. parahaemolyticus* (lacking the tdh and trh genes) can induce acute gastroenteritis in humans (Ottaviani et al., 2012). However, there was evidence of *V. parahaemolyticus* was isolated at 8.3% from acute diarrheal patients in the South of Vietnam in 2010, the present of *tdh* gene is 22.2% and the present of of trh gene is 19.4% (The Tai et al., 2011). In the late 1990s', V. parahaemolyticus was implicated in a large outbreak of the enteric disease in central Vietnam, the *tdh* gene was detected in 445 strains (85%) and the *trh* gene was detected in 6 strains (1.2%) (Chowdhury et al., 2004).

5. Conclusion

This study showed the high prevalence of *Vibrio* spp. in retail shrimp products. The AMR that existed among *Vibrio*. *V. parahaemolyticus* strain lacking virulence markers caused infection for the human. The results can be used as reference for more studies in the future. Therefore, more studies should be performed to have a better overview about virulence gene of shrimps. This article can be used as a reference source for public health officials to treat patients after digesting contaminated seafood. In addition, the farmers manage the disease treatment process to avoid exporting problems.

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