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Original Article

Characterization of *Vibrio parahaemolyticus* isolated from clinical specimens and oysters in Thailand

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Abstract

Introduction: *Vibrio parahaemolyticus* is a common pathogen that can cause seafood-borne gastroenteritis in humans. We determined the prevalence and characteristics of *V. parahaemolyticus* isolated from clinical specimens and oysters in Thailand.

Methodology: Isolates of *V. parahaemolyticus* from clinical specimens (n = 77) and oysters (n = 224) were identified by biochemical testing, polymerase chain reaction (PCR) assays, and serotyping. The toxin genes, antimicrobial resistance, and β-lactamase production were determined.

Results: A total of 301 isolates were confirmed as *V. parahaemolyticus* by PCR using specific primers for the *toxR* gene. The majority of clinical isolates carried the *tdh+/trh−* genotype (82.1%), and one of each isolate was *tdh−/trh+* and *tdh+/trh+* genotypes. One isolate from oyster contained the *tdh* gene and another had the *trh* gene. Twenty-six serotypes were characterized among these isolates, and O3:K6 was the most common (37.7%), followed by OUT:KUT, and O4:K9. In 2010, most clinical and oyster isolates were susceptible to antibiotics, with the exception of ampicillin. In 2012, clinical isolates were not susceptible to cephalothin (52.4%), streptomycin (95.2%), amikacin (66.6%), kanamycin (61.9%), and erythromycin (95.2%), significantly more frequently than in 2010. More than 95% of isolates that were not susceptible to ampicillin produced β-lactamase enzymes.

Conclusions: We found toxin genes in two oyster isolates, and the clinical isolates that were initially determined to be resistant to several antibiotics. Toxin genes and antimicrobial susceptibility profiles of *V. parahaemolyticus* from seafood and environment should be continually monitored to determine the spread of toxin and antimicrobial resistance genes.

Key words: *Vibrio parahaemolyticus*; serotype; oyster; Thailand.

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Introduction

Vibrio parahaemolyticus is a Gram-negative, motile halophilic bacterium that occurs in marine environments globally. Consumption of raw or undercooked seafood that is contaminated with virulent strains of *V. parahaemolyticus* can cause acute gastroenteritis [1]. In 2019, Thailand reported 110,736 cases of food poisoning, and laboratory testing of samples from 1,029 cases revealed *V. parahaemolyticus* in 245 patients (approximately 24%) [2]. According to a systematic review published in 2016, the concentration of *V. parahaemolyticus* was higher in oysters than in other seafood products [3] because oysters are filter feeders and concentrate this pathogen in their soft tissues. The incidence of gastroenteritis caused by *V. parahaemolyticus* associated with the consumption of oysters has been reported in the United States, Canada, China, Taiwan, Spain, Italy, Chile, Peru, and Brazil [4]. The Food and Agriculture Organization (FAO) reported that Asia had the highest oyster producing area, and Thailand ranked tenth among oyster producing countries [5]. In Thailand, it is common to consume fresh or partially cooked oysters for their freshness and flavor, and this contributes to increasing the risk of *V. parahaemolyticus* infection [6].

V. parahaemolyticus is divided into serotypes based on its lipopolysaccharide (O antigen) and capsular polysaccharide (K antigen) antigenic properties. Currently, commercial antisera are classified into 13 O types and 71 K types [7]. The first outbreak of the novel

serotype O3:K6 *V. parahaemolyticus* was reported in Kolkata, India, in 1996 [8]. Since then, the serotype O3:K6 and its serovariants (O4:K68, O1:K25, O1:KUT etc.) have been a public health concern worldwide, including in Thailand [9–10]. In general, serotype O3:K6 has the characteristics of a pandemic strain. It is positive for the *tdh* gene, and shares specific genetic markers including a distinctive *toxRS* [11] with orf8 [12]. Moreover, the serovariants of pandemic strains have identical profiles during molecular typing, and belong to sequence type (ST) 3 using multilocus sequence typing (MLST) analysis [13].

The pathogenesis of *V. parahaemolyticus* in humans is related to the production of three distinct toxins: thermostable direct hemolysin (TDH), thermostable direct-related hemolysin (TRH), and thermolabile hemolysin (TLH). The *tdh* gene, which encodes the pore-forming TDH, has several biological functions, including hemolytic activity, enterotoxicity, cytotoxicity, and cardiotoxicity [14]. *V. parahaemolyticus* strains that carry the *tdh* gene have been shown to induce complete hemolysis on Wagatsuma agar, which is referred to as the Kanagawa phenomenon (KP) [15]. TRH is encoded by the *trh* gene, which has 70% homology with the *tdh* gene. Similar to TDH, TRH exhibits hemolytic activity. The *tlh* gene, which encodes TLH, is a species-specific marker for *V. parahaemolyticus* identification by molecular detection methods [16]. TLH causes blood cell lysis and has phospholipase activity [17]. The majority of *V. parahaemolyticus* clinical strains carry the *tdh* and/or *trh* gene(s). In contrast, the prevalence of *tdh* and/or *trh* gene(s) among *V. parahaemolyticus* environmental strains were reported in 1–10%, depending on geographic area, sample source, and detection method [14]. Between 2006 to 2010, 51.3–73.6% of *V. parahaemolyticus* isolated from 776 patients in Southern Thailand were *tdh*+ *trh*− and group-specific polymerase chain reaction (PCR) positive pandemic strains [18]. The occurrence of *tdh* and *trh* genes among *V. parahaemolyticus* isolated from raw retail oysters in Thailand was 5.8% and 0.8%, respectively [19].

In general, mild cases of *V. parahaemolyticus* infection do not require treatment with antibiotic therapy. In cases with severe or prolonged illness, the recommended antibiotics for treatment are cephalosporins, tetracyclines, and fluoroquinolones (e.g., ciprofloxacin) [20]; however, antimicrobial susceptibility testing should be done prior to selecting a medication. In the past few decades, several studies have reported the emergence of antibiotic-resistant *V. parahaemolyticus* due to overuse and abuse of antibiotics in humans, agriculture, and aquaculture [20-22]. Therefore, antimicrobial susceptibility testing of *V. parahaemolyticus* isolates from clinical and environmental samples should be performed to monitor changes in bacterial resistance to antimicrobial agents, particularly those used for first-line treatment of infections [22].

In Thailand, a number of studies have previously investigated the molecular characteristics, epidemiological data, and pathogenicity of *V. parahaemolyticus* strains in humans, marine bivalves, and the environment [10,18,23]. To further advance understanding in this area, we determined the prevalence of the toxin genes, serotypes, and antimicrobial susceptibility of *V. parahaemolyticus* from clinical and oyster isolates collected in 2010 and 2012 in Thailand.

Methodology

Bacterial strains

The current study investigated 77 *V. parahaemolyticus* clinical isolates collected during outbreaks in 2010 (n = 56) and 2012 (n = 21), and 224 *V. parahaemolyticus* isolates from oysters collected in Thailand during April–May 2010. The oysters were collected from oyster farms in Kanchanadit, Surat Thani province, and Ang Sila, Chonburi province. Reference strains for this study included *V. parahaemolyticus* ATCC17802, *V. parahaemolyticus* AQ4613, and *V. parahaemolyticus* AQ902. The isolates were kept in Luria Bertani (LB) broth containing 1% sodium chloride (NaCl) and 60% glycerol at −80 °C, in the microbiology laboratory, Faculty of Medical Technology, Rangsit University.

This study did not use medical records or archived specimens. Specimens were not collected for this study, and the microbial strains were obtained from microbiological laboratories. The study was approved by the Ethics Review Board of Rangsit University (RSEC 04/53). The board waived the need for consent because patients' data were completely anonymous.

Bacterial identification

All isolates were identified as *V. parahaemolyticus* using conventional biochemical tests according to the United States Federal Drug Agency (USFDA) Bacteriological Analytical Manual [24]. These tests included oxidase, triple sugar iron agar, motility, arginine dihydrolase, ornithine, and lysine decarboxylase, Voges-Proskauer, Simmon's citrate test, arabinose, lactose, and mannitol fermentation.

Urea agar was used to determine the ability of the bacteria to produce urease. Nutrient broths with 0%, 2%, 6%, 8%, and 10% NaCl were used for salt tolerance testing. *V. parahaemolyticus* was confirmed using PCR assay and amplification of the 368 bp fragment of the *toxR* gene [25,26]. This gene is crucial to *V. parahaemolyticus* virulence and is well conserved across strains.

Serotyping

V. parahaemolyticus was characterized using a commercial *V. parahaemolyticus* antisera test kit (Denka Seiken, Tokyo, Japan). The isolates were grown overnight on LB agar plates (BD Difco, Franklin Lakes, USA) containing 3% NaCl. Cell suspensions that were autoclaved at 121 °C for 30 minutes were used for O grouping, and non-autoclaved suspensions were used for K antigen typing. O grouping and K typing were performed using the slide agglutination method according to the manufacturer's instructions.

Detection of TDH by KP test

The KP test was used to determine TDH production based on a previously reported protocol [27]. *V. parahaemolyticus* isolates were cultured on Wagatsuma agar medium that contained 3 g of yeast extract, 10 g of peptone, 70 g of NaCl, 5 g of K_2HPO_4, 10 g of mannitol, 0.001 g of crystal violet, 15 g of agar, 1 L of distilled water, and 50 mL of sheep/human anticoagulated blood. KP tests were scored as positive for *V. parahaemolyticus* isolates that displayed a distinctive halo around colonies due to β-hemolysis after an overnight incubation at 37 °C. *V. parahaemolyticus* strains AQ4613 and ATCC17802 were used as positive and negative controls, respectively.

Detection of toxin genes by multiplex PCR

The optimized boiling method was used to extract the genomic DNA of *V. parahaemolyticus* for PCR assay [26]. The virulence genes *tlh*, *tdh*, and *trh* were detected using multiplex PCR in previous reports [28-30]. *V. parahaemolyticus* AQ902 strain (*tlh*+, *tdh*+, and *trh*+) was used as the positive control. The details of the oligonucleotide primers, sizes of PCR amplified products, and PCR conditions are presented in Supplementary Table 1. MyCycler Thermal Cycler (BiORAD, Hercules, USA) was used for PCR. The PCR mix contained 5 µL of 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTPs, 0.25 µM of each primer, 0.5 U of i-*Taq* DNA polymerase (iNtRON Biotechnology, Inc., Gyeonggi-do, South Korea), 1 µL of DNA template, and DNase-free H_2O to a final volume of 25 µL. Five microliter samples of PCR products were separated using 1.5% agarose gel electrophoresis with a 100 bp DNA ladder (Promega, Madison, USA) and visualized on a UV trans-illuminator.

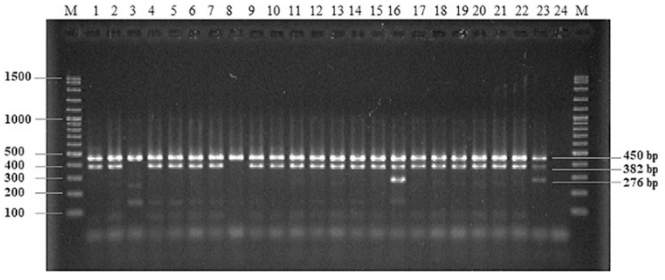
Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was conducted using the agar disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [31]. Thirteen different antibiotic disks (Oxoid, Hampshire, UK) were used for the tests: ampicillin (10 µg), cephalothin (30 µg), ceftriaxone (30 µg), gentamicin (10 µg), streptomycin (10 µg), amikacin (30 µg), kanamycin (30 µg), erythromycin (15 µg), tetracycline (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), and chloramphenicol (30 µg). *Staphylococcus aureus* ATCC25923 and *Escherichia coli* ATCC25922 were used as reference strains for quality control of the assay.

Detection of β-lactamase enzymes

β-lactamase detection was performed using the commercially available cefinase discs test kit (BD BBL, Franklin Lakes, USA). The cefinase discs are impregnated with nitrocefin, a chromogenic cephalosporin. When β-lactamase hydrolyzes the amide bond in the β-lactam ring of this compound, the color rapidly changes from yellow to red, indicating bacterial production of this enzyme in significant amounts. The bacteria used as positive and negative controls were *Staphylococcus aureus* ATCC29213 and *Escherichia coli* ATCC25922, respectively.

Figure 1. Detection of virulence associated with gene in *V. parahaemolyticus* strains by multiplex PCR, electrophoresed on 1.5% (w/v) agarose gel.



Lane M, 100-base pair plus ladder (Promega, Madison, USA); Lanes 1, 2, 4–7, 9–15, and 17–22 (tlh+/tdh+/trh−); Lanes 3 and 8 (tlh+/tdh−/trh−); Lane 16, (tlh+/tdh+/trh+); Lane 23, *V. parahaemolyticus* AQ902 as the positive control containing the tlh (450 bp), tdh (382 bp), and trh (276 bp) genes; Lane 24, negative control.

Statistical analysis

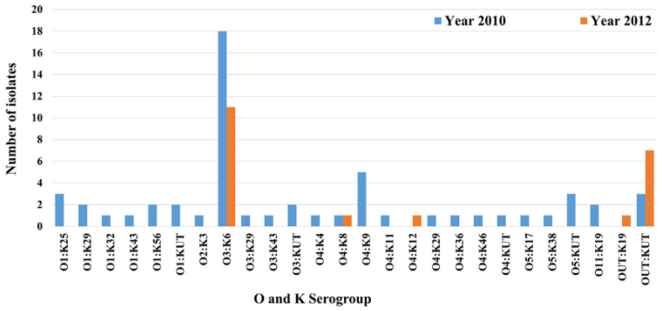
Chi square or Fisher's exact test were used to analyze the relationship between antimicrobial susceptibility, serogroup, and year of isolation, as well as the association between the toxin genes and bacterial sources. The results were considered statistically significant if the *p* value was less than 0.05. SPSS version 21 software (IBM SPSS Inc., NY) was used for all statistical analyses.

Results

V. parahaemolyticus was confirmed in 301 isolates from clinical specimens and oysters using PCR assay to detect the *toxR* gene. Multiplex PCR was used to successfully amplify the toxin genes of 224 oyster and 56 clinical *V. parahaemolyticus* isolates in 2010 (Figure 1). The *tlh* gene (280, 100.0%) was the most common toxin gene, followed by *tdh* (48, 17.1%), and *trh* (3, 1.1%). The prevalence of *tdh* and *trh* genes was significantly higher in clinical isolates than in oyster isolates ($p < 0.001$ and $p < 0.05$, respectively; Table 1). The *tdh*+/*trh*− genotype was found to be significantly dominant in clinical isolates ($p < 0.001$), while the *tdh*−/*trh*− genotype was more commonly present in oyster isolates ($p < 0.001$). We also detected KP in 280 *V. parahaemolyticus* isolates using Wagatsuma agar. KP-positive isolates were found significantly more frequently in clinical isolates than in the other group ($p < 0.001$; Table 1).

Among the 77 clinical isolates of *V. parahaemolyticus* in 2010 and 2012, 26 different serotypes were identified (Figure 2). There was no statistically significant correlation between frequencies of *V. parahaemolyticus* serotypes and year of isolation. The serotype O3:K6 was predominantly found in both the 2010 (18/56 strains, 32.1%) and the 2012 (11/21 strains, 52.4%) outbreaks. The serotypes O1:K25, O4:K9, and O5:KUT were found among isolates in 2010. Two of the eight serogroup O strains for which the K antigens could not be typed were O1 (O1:KUT), two were O3 (O3:KUT), one was O4 (O4:KUT), and three were O5 (O5:KUT). The non-typeable strains for O and K antigens (OUT:KUT) in 2012 (7/21 strains, 33.3%) were slightly increased from 2010 (3/56 strains, 5.4%). The serotypes O4:K12 and OUT:K19 were found only in 2012.

Figure 2. Distribution of serotypes among clinical and environmental isolates of *V. parahaemolyticus* in 2010 and 2012.



All the serotype O3:K6 clinical isolates (18, 32.1%) carried the *tdh* gene, while some isolates of O1:K29, O1:K32, O1:KUT, O3:KUT, O4:K29, O5:K17, O11:K19, and OUT (8, 14.3%) did not have either the *tdh* or the *trh* gene (Table 2). Of the 46 KP-positive *V. parahaemolyticus* isolates, 45 carried *tdh*, including one strain that had both *tdh* and *trh*. In contrast, two strains that were KP negative carried *tdh* or *trh*. Two strains (3.6%) were positive for urease production and possessed the *trh* gene. Only one O1:KUT isolate tested positive for both the *tdh* and *trh* genes (Table 3). Two oyster isolates that contained the *tdh* or *trh* gene had O3:K6 and O4:K34 serotypes, respectively (Table 2).

The antimicrobial susceptibility patterns of 301 *V. parahaemolyticus* isolates from clinical samples and oysters—77 clinical isolates (56 in 2010 and 21 in 2012) and 224 oyster isolates in 2010—are listed in Table 3.

Table 1. Distribution of virulence factors in clinical and oyster isolates of *V. parahaemolyticus* in Thailand in 2010.

Virulence factors	No. of isolates (%)		*p* values
	Clinical (N = 56)	Oyster (N = 224)	
Toxin gene			
tlh	56 (100)	224 (100)	
tdh	47 (83.9)	1 (0.5)	< 0.001
trh	2 (3.6)	1 (0.5)	0.042
Toxin gene profiles			
tdh^+, trh^+	1 (1.8)	0 (0.0)	
tdh^+, trh^-	46 (82.1)	1 (0.5)	< 0.001
tdh^-, trh^+	1 (1.8)	1 (0.5)	0.287
tdh^-, trh^-	8 (14.3)	222 (99.1)	< 0.001
Others			
KP [a]	46 (82.1)	1 (0.5)	< 0.001
Urease	2 (3.6)	2 (0.9)	0.130
β - lactamase	54 (96.4)	205 [b] (95.8)	0.831

[a] KP: Kanagawa phenomenon; [b] 214 oyster isolates were tested for β - lactamase production by cefinase test.

Table 2. Serotypes and virulence factors of 280 clinical and oyster isolates of *V. parahaemolyticus* in Thailand in 2010.

O serogroup	Serotype	No. of isolate (s)	Virulence index					
			tlh	*tdh*	*trh*	KP [a]	Urease	β - lactamase
***V. parahaemolyticus* isolated from human patients (N = 56)**								
O1	O1:K25	3	+	+	-	+	-	+
	O1:K29	1	+	-	-	-	-	+
		1	+	+	-	+	-	+
	O1:K32	1	+	-	-	-	-	+
	O1:K43	1	+	+	-	+	-	+
	O1:K56	2	+	+	-	+	-	+
	O1:KUT	1	+	+	+	+	+	+
		1	+	-	-	-	-	+
O2	O2:K3	1	+	+	-	+	-	+
O3	O3:K6	18	+	+	-	+	-	+
	O3:K29	1	+	+	-	+	-	+
	O3:K43	1	+	+	-	+	-	+
	O3:KUT	1	+	-	-	-	-	+
		1	+	-	+	-	+	+
O4	O4:K4	1	+	+	-	+	-	+
	O4:K8	1	+	+	-	+	-	+
	O4:K9	4	+	+	-	+	-	+
		1	+	+	-	+	-	-
	O4:K11	1	+	+	-	+	-	+
	O4:K29	1	+	-	-	-	-	+
	O4:K36	1	+	+	-	+	-	+
	O4:K46	1	+	+	-	+	-	+
	O4:KUT	1	+	+	-	+	-	+
O5	O5:K17	1	+	-	-	-	-	-
	O5:K38	1	+	+	-	+	-	+
	O5:KUT	3	+	+	-	+	-	+
O11	O11:K19	1	+	-	-	-	-	+
		1	+	+	-	-	-	+
OUT		2	+	+	-	+	-	+
		1	+	-	-	-	-	+
***V. parahaemolyticus* isolated from oyster (N = 224)**								
O3	O3:K6	1	+	+	-	+	-	+
O4	O4:K34	1	+	-	+	-	-	+
ND [b]	ND	2	+	-	-	-	+	+
ND	ND	201	+	-	-	-	-	+
ND	ND	9	+	-	-	-	-	-
ND	ND	10	+	-	-	-	-	ND

tlh: thermolabile haemolysin gene; *tdh*: thermostable direct haemolysin gene; *trh*: thermostable-related haemolysin gene; KP: Kanagawa phenomenon; +: positive; -: negative; ND: not done.

Table 3. Antimicrobial susceptibility patterns of 301 clinical and oyster isolates of *V. parahaemolyticus* in Thailand.

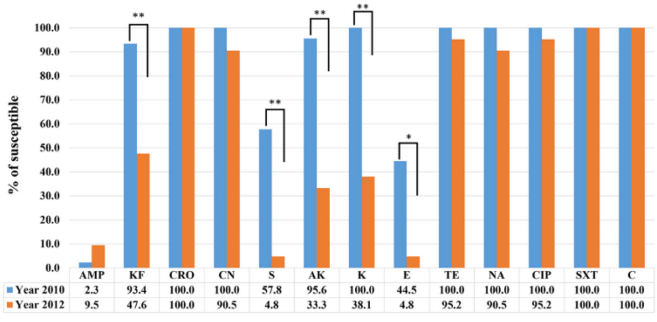
Amp	TE	CIP	CRO	SXT	C	CN	No. of isolates (%)		
							Clinical in 2010 (N = 56)	**Clinical in 2012 (N = 21)**	**Oyster in 2010 (N = 224)**
R	S	S	S	S	S	S	36 (64.3)	12 (57.1)	154 (68.8)
I	S	S	S	S	S	S	18 (32.1)	4 (19.1)	61 (27.2)
S	S	S	S	S	S	S	1 (1.8)	2 (9.5)	5 (2.2)
S	R	S	S	S	S	R	0 (0.0)	3 (14.3)	0 (0.0)
R	S	S	S	S	S	I	0 (0.0)	1 (4.7)	2 (0.9)
R	S	I	S	S	S	S	0 (0.0)	2 (9.5)	0 (0.0)
I	S	I	S	S	S	I	0 (0.0)	1 (4.7)	0 (0.0)
R	I	I	S	S	S	I	0 (0.0)	0 (0.0)	1 (0.4)
R	S	S	I	S	S	S	0 (0.0)	0 (0.0)	1 (0.4)

Amp: ampicillin; TE: tetracycline; CIP: ciprofloxacin; CRO: ceftriaxone; SXT: trimethoprim-sulfamethoxazole; C: chloramphenicol; CN: gentamicin.

Our study found nine different microbial susceptibility patterns. The most common pattern among clinical and oyster isolates was A, which indicated resistance to ampicillin only. Among the *V. parahaemolyticus* isolates from clinical specimens in 2010, 54 were non-susceptible (resistant or intermediate) to ampicillin and positive for β-lactamase production (96.4%), while two isolates that were β-lactamase negative were susceptible to ampicillin. A total of 205 *V. parahaemolyticus* isolates from oysters produced β-lactamase and were non-susceptible to ampicillin. There was no statistically significant difference in the frequency of β-lactamase-producing strains in clinical isolates versus oyster isolates (Table 1). The antimicrobial susceptibility patterns D to I were associated with resistance or intermediate resistance to other antimicrobial agents, with the exceptions of trimethoprim-sulfamethoxazole and chloramphenicol.

We compared the proportion of antimicrobial susceptibility of *V. parahaemolyticus* isolates from clinical specimens between 2010 and 2012. Among the 66 isolates tested, 100% were susceptible to ceftriaxone, trimethoprim-sulfamethoxazole, and chloramphenicol. Conversely, ampicillin resistance was common among these *V. parahaemolyticus* strains (44, 66.7%). Forty-three isolates (65.2%) showed intermediate erythromycin susceptibility. We compared the percentage of clinical isolates susceptible to antimicrobial agents between 2010 and 2012. Interestingly, the rates for susceptibility to cephalothin, streptomycin, amikacin, and kanamycin among *V. parahaemolyticus* isolated in 2012 were significantly lower than those isolated in 2010 ($p < 0.0001$; Figure 3), and the same pattern was seen for erythromycin ($p = 0.001$).

Figure 3. Antimicrobial susceptibility among clinical isolates of *V. parahaemolyticus*.



Significant differences in the percentage of susceptibility between 2010 and 2012 strains: *p = 0.001 and **p < 0.0001. AMP: ampicillin; KF: cephalothin; CRO: ceftriaxone; CN: gentamicin; S: streptomycin; AK: amikacin; K: kanamycin; E: erythromycin; E: tetracycline; NA: nalidixic acid; CIP: ciprofloxacin; SXT: trimethoprim-sulfamethoxazole; C: chloramphenicol.

Discussion

Consumption of seafood contaminated with *V. parahaemolyticus* is a major cause of gastroenteritis. This Gram-negative bacterium thrives in salt conditions and is distributed throughout temperate and tropical regions worldwide. *V. parahaemolyticus* is often found in coastal areas, seawater, sediments, and marine animals, especially shrimp, shellfish, crabs, and fish [32]. Thai people prefer to consume raw or undercooked oysters, which carries a risk of seafood poisoning due to *V. parahaemolyticus*. We characterized the distribution of toxin gene profiles of *V. parahaemolyticus* isolates from clinical specimens and oysters. More than 80% of the clinical isolates harbored only the *tdh* gene, and only a few carried the *trh* gene, as has been reported in other countries [10,33-34]. Among the *tdh*-positive isolates in this study, only one did not show KP, implying that KP is not a sensitive indicator for differentiating pathogenic and non-pathogenic *V. parahaemolyticus* strains [35]. In addition, we found that only one of the clinical isolates had the *tdh*+/*trh*+ genotype, which was consistently observed among clinical and cockle isolates in Thailand [6]. The oyster isolates in the present study exhibited the *tdh*−/*trh*− genotype in a significantly higher proportion, as was also reported previously in the United States [33]. One isolate from oyster contained the *tdh* gene and another exhibited the *trh* gene, indicating the risk of consuming raw or undercooked oysters. According to the USFDA, consuming 10,000 *V. parahaemolyticus* cells in a serving of oysters, or around 50 cells/g of oysters, can cause illness. Intervention strategies to reduce or decontaminate the number of *V. parahaemolyticus* in raw oysters are required. Various techniques for decontamination of *V. parahaemolyticus* without sacrificing the flavor or nutritional value of raw oysters have been developed. These techniques include physical methods (icing, immediate refrigeration, freezing, depuration, relaying and transplanting, irradiation, and high-pressure processing) and chemical methods (use of natural antimicrobial agents) [4]. Our findings of a low prevalence of *V. parahaemolyticus* (*tdh*+ or *trh*+) in oyster isolates were consistent with previous reports on environmental isolates [36–39]. The *tdh*−/*trh*− genotype was found in 14.3% clinical isolates, and these strains could possibly contain other virulence-associated genes such as type III secretion systems 1 and 2 (*vscC2*, *vopB2*, *vscS2*, *vopC*, *vopA* genes etc.)

[1,35]. Therefore, other virulence factors of *V. parahaemolyticus* should be investigated to better understand the exact mechanism of infection and host-pathogen interactions.

Our findings showed that 3.6% of the clinical isolates that produced urease also carried the *trh* gene, confirming that the urease-positive phenotype is significantly correlated with the presence of the *trh* gene, which is considered a typical virulence indicator for *V. parahaemolyticus* [8,40]. One *trh*-positive oyster isolate failed to produce urease; this isolate may not have expressed the gene for enzyme production under our experimental conditions. The *trh* gene was not found in two urease-positive oyster isolates, which was similar to the results of Kongrueng *et al.* [41], who reported that the *trh* gene was not involved in urease production among *trh*-positive *V. parahaemolyticus*.

Our study found a high level of serotype diversity (26 serotypes), with O3:K6 (29/77, 37.7%) being the most common, followed by OUT:KUT (10/77, 13.0%), and O4:K9 (5/77, 6.5%). In contrast to the clinical isolates from 2012 (five serotypes), the clinical isolates from 2010 were extremely diverse, with 24 different types identified. The serotype O4:K9 was not found in 2012, and O4:K12 and OUT:K19 appeared only in this year. The changes in serotypes of *V. parahaemolyticus* occur due to a frequent recombination event between the O and K antigen encoding gene clusters [42]. In addition, the difference of the serotypes present in *V. parahaemolyticus* in any region may be associated with differences in environmental parameters, such as temperature and acidification [43]. Among the oyster isolates, one isolate of *tdh*-positive was serotype O3:K6, which was the dominant serotype found among the clinical isolates. The serotype O4:K34 *trh*-positive was found only in one oyster isolate. Consistently, the serotypes O3:K6 and O4:K34 were previously reported from oyster isolates in the United States [33] and seafood isolates in China [44].

In the current study, the majority (> 90%) of clinical and oyster isolates of *V. parahaemolyticus* collected in 2010 were susceptible to all antimicrobial agents, except for ampicillin. This finding is consistent with reports from Thailand [10] and China [45]. Our findings revealed no significant difference between the isolation sources and antimicrobial susceptibility patterns. Among the ampicillin non-susceptible isolates, more than 95% produced β-lactamase. In addition, one clinical and 10 oyster isolates did not produce β-lactamase but were resistant to ampicillin, which suggests that *V. parahaemolyticus* may have other resistance mechanisms, such as decreased drug uptake, drug target modification, or drug efflux [46].

We monitored the antimicrobial agent susceptibility of *V. parahaemolyticus* isolates from clinical specimens in 2012. We observed that more than 90% of *V. parahaemolyticus* isolates were still resistant to ampicillin. This finding indicates that ampicillin may be not suitable for treating *V. parahaemolyticus* infections. Moreover, we found that the clinical isolates in 2012 were significantly resistant to cephalosporin (cephalothin), aminoglycosides (streptomycin, amikacin, kanamycin), and macrolide (erythromycin), in agreement with a study in Chile [47]. The percentages of isolates susceptible to gentamicin (90.5%) and amikacin (33.3%) in this study were relatively low compared with a report from China, at 97.5% and 98%, respectively [45]. However, 95.2% of clinical isolates were susceptible to tetracycline and ciprofloxacin, suggesting that the current treatment options for *V. parahaemolyticus* infection are effective.

The limitation of this study is that the *V. parahaemolyticus* isolates were fairly old; the new strains from clinical setting and oysters should be further collected and analyzed.

Conclusions

We found that two oyster isolates carried the *tdh* or *trh* gene, implying that toxin genes in *V. parahaemolyticus* from seafood and the environment should be monitored. Most *V. parahaemolyticus* isolates from clinical setting and oysters were found to be resistant to ampicillin due to β-lactamase production. In addition, some of the clinical isolates were initially found to be resistant to several antibiotics, hence the antimicrobial susceptibility test of organisms isolated from humans and the environment should be continually determined for monitoring the spread of antimicrobial resistance. However, tetracycline and ciprofloxacin are still suitable for treatment of the patients infected with *V. parahaemolyticus*.

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References

1. Ghenem L, Elhadi N, Alzahrani F, Nishibuchi M (2017) *Vibrio parahaemolyticus*: a review on distribution, pathogenesis,

virulence determinants and epidemiology. Saudi J Med Med Sci 2: 93–103. doi: 10.4103/sjmms.sjmms_30_17.
2. Department of Disease Control, Thailand Ministry of Public Health (2019) Epidemiological surveillance report 2019. Available: https://ddc.moph.go.th/uploads/publish/1129820210620030431.pdf. Accessed: 7 January 2023.
3. Odeyemi OA (2016) Incidence and prevalence of *Vibrio parahaemolyticus* in seafood: a systematic review and meta-analysis. Springerplus 5: 464. doi: 10.1186/s40064-016-2115-7.
4. Ndraha N, Wong HC, Hsiao HI (2020) Managing the risk of *Vibrio parahaemolyticus* infections associated with oyster consumption: a review. Compr Rev Food Sci Food Saf 19: 1187–1217. doi: 10.1111/1541-4337.12557.
5. Food and Agriculture Organization (FAO) of the United Nations (2019) Fisheries and aquaculture information and statistics branch. Available: http://www.fao.org/fishery/statistics/globalproduction/en. Accessed: 15 March 2023.
6. Changchai N, Saunjit S (2014) Occurrence of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in retail raw oysters from the eastern coast of Thailand. Southeast Asian J Trop Med Public Health 45: 662–669.
7. Oliver JD, Jones JL (2015) *Vibrio parahaemolyticus* and *Vibrio vulnificus*. In Tang YW, Sussman M, Liu D, Poxton I, Schwartzman J, editors. Molecular Medical Microbiology, 2nd Edition. London: Academic Press. 1169–1186. doi: 10.1016/B978-0-12-397169-2.00066-4.
8. Okuda J, Ishibashi M, Hayakawa E, Nishino T, Takeda Y, Mukhopadhyay AK, Garg S, Bhattacharya SK, Nair GB, Nishibuchi M (1997) Emergence of a unique O3:K6 clone of *Vibrio parahaemolyticus* in Calcutta, India, and isolation of strains from the same clonal group from Southeast Asian travelers arriving in Japan. J Clin Microbiol 12: 3150–3155. doi: 10.1128/jcm.35.12.3150-3155.1997.
9. Laohaprertthisan V, Chowdhury A, Kongmuang U, Kalnauwakul S, Ishibashi M, Matsumoto C, Nishibuchi M (2003) Prevalence and serodiversity of the pandemic clone among the clinical strains of *Vibrio parahaemolyticus* isolated in southern Thailand. Epidemiol Infect 130: 395–406. doi: 10.1017/S0950268803008458.
10. Mala W, Alam M, Angkititrakul S, Wongwajana S, Lulitanond V, Huttayananont S, Kaewkes W, Faksri K, Chomvarin C (2016) Serogroup, virulence, and molecular traits of *Vibrio parahaemolyticus* isolated from clinical and cockle sources in northeastern Thailand. Infect Genet Evol 39: 212–218. doi: 10.1016/j.meegid.2016.01.006.
11. Matsumoto C, Okuda J, Ishibashi M, Iwanaga M, Garg P, Rammamurthy T, Wong HC, Depaola A, Kim YB, Albert MJ, Nishibuchi M (2000) Pandemic spread of an O3:K6 clone of *Vibrio parahaemolyticus* and emergence of related strains evidenced by arbitrarily primed PCR and *toxRS* sequence analyses. J Clin Microbiol 38: 578–585. doi: 10.1128/JCM.38.2.578-585.2000.
12. Nasu H, Iida T, Sugahara T, Yamaichi Y, Park KS, Yokoyama K, Makino K, Shinagawa H, Honda T (2000) A filamentous phage associated with recent pandemic *Vibrio parahaemolyticus* O3:K6 strains. J Clin Microbiol 38: 2156–2161. doi: 10.1128/JCM.38.6.2156-2161.2000.
13. Han D, Yu F, Tang H, Ren C, Wu C, Zhang P, Han C (2017) Spreading of pandemic *Vibrio parahaemolyticus* O3:K6 and its serovariants: a re-analysis of strains isolated from multiple studies. Front Cell Infect Microbiol 7: 188. doi: 10.3389/fcimb.2017.00188.
14. Raghunath P (2015) Roles of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) in *Vibrio parahaemolyticus*. Front Microbiol 5: 805. doi: 10.3389/fmicb.2014.00805.
15. Shimohata T, Takahashi A (2010) Diarrhea induced by infection of *Vibrio parahaemolyticus*. J Med Invest 57: 179–182. doi: 10.2152/jmi.57.179.
16. Klein SL, Gutierrez West CK, Mejia DM, Lovell CR (2014) Genes similar to the *Vibrio parahaemolyticus* virulence-related genes *tdh*, *tlh*, and *vscC2* occur in other Vibrionaceae species isolated from a pristine estuary. Appl Environ Microbiol 80: 595–602. doi: 10.1128/AEM.02895-13.
17. Broberg CA, Calder TJ, Orth K (2011) *Vibrio parahaemolyticus* cell biology and pathogenicity determinants. Microbes Infect 13: 992–1001. doi: 10.1016/j.micinf.2011.06.013.
18. Thongjun J, Mittraparp-Arthorn P, Yingkajorn M, Kongreung J, Nishibuchi M, Vuddhakul V (2013) The trend of *Vibrio parahaemolyticus* infections in Southern Thailand from 2006 to 2010. Trop Med Health 41: 151–156. doi: 10.2149/tmh.2013-06.
19. Changchai N, Saunjit S (2014) Occurrence of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in retail raw oysters from the eastern coast of Thailand. Southeast Asian J Trop Med Public Health 45: 662–669.
20. Han F, Walker RD, Janes ME, Prinyawiwatkul W, Ge B (2007) Antimicrobial susceptibilities of *Vibrio parahaemolyticus* and *Vibrio vulnificus* isolates from Louisiana Gulf and retail raw oysters. Appl Environ Microbiol 21: 7096–7098. doi: 10.1128/AEM.01116-07.
21. Cabello FC, Godfrey HP, Tomova A, Ivanova L, Dölz H, Millanao A, Buschmann AH (2013) Antimicrobial use in aquaculture re-examined: its relevance to antimicrobial resistance and to animal and human health. Environ Microbiol 7: 1917–1942. doi: 10.1111/1462-2920.12134.
22. Elmahdi S, DaSilva LV, Parveen S (2016) Antibiotic resistance of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in various countries: a review. Food Microbiol 57: 128–134. doi: 10.1016/j.fm.2016.02.008.
23. Theethakaew C, Feil EJ, Castillo-Ramírez S, Aanensen DM, Suthienkul O, Neil DM, Davies RL (2013) Genetic relationships of *Vibrio parahaemolyticus* isolates from clinical, human carrier, and environmental sources in Thailand, determined by multilocus sequence analysis. Appl Environ Microbiol 7: 2358–2370. doi: 10.1128/AEM.03067-12.
24. Elliot EL, Kaysner AC, Jackson L, Tamplin ML (2001) *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and other *Vibrio* spp. In USFDA bacteriological analytical manual. 8th Edition. Maryland: AOAC International, Gaithersburg. 901–927.
25. Kim YB, Okuda J, Matsumoto C, Takahashi N, Hashimoto S, Nishibuchi M (1999) Identification of *Vibrio parahaemolyticus* strains at the species level by PCR targeted to the *toxR* gene. J Clin Microbiol 4: 1173–1177. doi: 10.1128/JCM.37.4.1173-1177.1999.
26. Guin S, Saravanan M, Anjay, Chowdhury G, Pazhani GP, Ramamurthy T, Chandra Das S (2019) Pathogenic *Vibrio parahaemolyticus* in diarrhoeal patients, fish and aquatic environments and their potential for inter-source transmission. Heliyon 5: e01743. doi: 10.1016/j.heliyon.2019.e01743.
27. Canizalez-Roman A, Flores-Villaseñor H, Zazueta-Beltran J, Muro-Amador S, León-Sicairos N (2011) Comparative

evaluation of a chromogenic agar medium-PCR protocol with a conventional method for isolation of *Vibrio parahaemolyticus* strains from environmental and clinical samples. Can J Microbiol 57: 136–142. doi: 10.1139/W10-108.
28. Bej AK, Patterson DP, Brasher CW, Vickery MCL, Jones DD, Kaysner CA (1999) Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh* and *trh*. J Microbiol Methods 36: 215–225. doi: 10.1016/S0167-7012(99)00037-8.
29. Nishibuchi M, Taniguchi T, Misawa T, Khaeomanee-IAM V, Honda T and Miwatani T (1989) Cloning and nucleotide sequence of the genetic encoding the hemolysin related to the thermostable direct hemolysin of *Vibrio parahaemolyticus*. Infect. Immun 57: 2691–2697. doi: 10.1128/iai.57.9.2691-2697.1989.
30. Nishibuchi M, Kaper JB (1990) Duplication and variation of the *thermostable direct haemolysin* (*tdh*) gene in *Vibrio parahaemolyticus*. Mol Microbiol 4: 87–99. doi: 10.1111/j.1365-2958.1990.tb02017.x.
31. Clinical and Laboratory Standards Institute (2015) Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria. M45. 3th edition. Wayne, PA.
32. Letchumanan V, Chan KG, Lee LH (2014) *Vibrio parahaemolyticus*: a review on the pathogenesis, prevalence, and advance molecular identification techniques. Front Microbiol 5: 705. doi: 10.3389/fmicb.2014.00705.
33. Jones JL, Lüdeke CH, Bowers JC, Garrett N, Fischer M, Parsons MB, Bopp CA, DePaola A (2012) Biochemical, serological, and virulence characterization of clinical and oyster *Vibrio parahaemolyticus* isolates. J Clin Microbiol 7: 2343–2352. doi: 10.1128/JCM.00196-12.
34. Tsai SE, Jong KJ, Tey YH, Yu WT, Chiou CS, Lee YS, Wong HC (2013) Molecular characterization of clinical and environmental *Vibrio parahaemolyticus* isolates in Taiwan. Int J Food Microbiol 1: 18–26. doi: 10.1016/j.ijfoodmicro.2013.04.017.
35. Wang R, Zhong Y, Gu X, Yuan J, Saeed AF, Wang S (2015) The pathogenesis, detection, and prevention of *Vibrio parahaemolyticus*. Front Microbiol 6: 144. doi: 10.3389/fmicb.2015.00144.
36. Almuhaideb E, Chintapenta LK, Abbott A, Parveen S, Ozbay G (2020) Assessment of *Vibrio parahaemolyticus* levels in oysters (*Crassostrea virginica*) and seawater in Delaware Bay in relation to environmental conditions and the prevalence of molecular markers to identify pathogenic *Vibrio parahaemolyticus* strains. PLoS One 12: e0242229. doi: 10.1371/journal.pone.0242229.
37. Yang Y, Xie J, Li H, Tan S, Chen Y, Yu H (2017) Prevalence, antibiotic susceptibility and diversity of *Vibrio parahaemolyticus* isolates in seafood from South China. Frontiers in Microbiology 8: 2566. doi: 10.3389/fmicb.2017.02566.
38. Rojas MVR, Matte' MH, Dropa M, Silva MLD, Matte' GR (2011) Characterization of *Vibrio parahaemolyticus* isolated from oysters and mussels in São Paulo, Brazil. Revista do Instituto de Medicina Tropical de São Paulo 4: 201–205. doi: 10.1590/S0036-46652011000400005.
39. Wagley S, Koofhethile K, Wing JB, Rangdale R (2008) Comparison of *V. parahaemolyticus* isolated from seafoods and cases of gastrointestinal disease in the UK. Int J Environ Health Res 4: 283–293. doi: 10.1080/09603120801911064.
40. Suthienkul O, Ishibashi M, Iida, T, Nettip N, Supavej S, Eampokatap B, Makino M. and Honda T (1995) Urease production correlates with possession of the trh gene in *Vibrio parahaemolyticus* strains isolated in Thailand. J Infect Dis 172: 1405–1408. doi: 10.1093/infdis/172.5.1405.
41. Kongrueng J, Srinitiwarawong K, Nishibuchi M, Mittraparp-Arthorn P, Vuddhakul V (2018) Characterization and CRISPR-based genotyping of clinical *trh*-positive *Vibrio parahaemolyticus*. Gut Pathog 10: 48. doi: 10.1186/s13099-018-0275-4.
42. Chen Y, Stine OC, Badger JH, Gil AI, Nair GB, Nishibuchi M, Fouts DE (2011) Comparative genomic analysis of *Vibrio parahaemolyticus*: serotype conversion and virulence. BMC Genomics. 12: 294. doi: 10.1186/1471-2164-12-294.
43. Nair GB, Ramamurthy T, Bhattacharya SK, Dutta B, Takeda Y, Sack DA (2007) Global dissemination of *Vibrio parahaemolyticus* serotype O3:K6 and its serovariants. Clin Microbiol Rev 20: 39–48. doi: 10.1128/CMR.00025-06.
44. Chen X, Zhu Q, Yu F, Zhang W, Wang R, Ye X, Jin L, Liu Y, Li S, Chen Y (2018) Serology, virulence and molecular characteristics of *Vibrio parahaemolyticus* isolated from seafood in Zhejiang province. PLoS One 10: e0204892. doi: 10.1371/journal.pone.0204892.
45. Chen Y, Chen X, Yu F, Wu M, Wang R, Zheng S, Han D, Yang Q, Kong H, Zhou F, Zhu J, Yao H, Zhou W, Li L (2016) Serology, virulence, antimicrobial susceptibility and molecular characteristics of clinical *Vibrio parahaemolyticus* strains circulating in southeastern China from 2009 to 2013. Clin Microbiol Infect 3: 258.e9–16. doi: 10.1016/j.cmi.2015.11.003.
46. Reygaert WC (2018) An overview of the antimicrobial resistance mechanisms of bacteria. AIMS Microbiol 3: 482–501. doi: 10.3934/microbiol.2018.3.482.
47. Dauros P, Bello H, Domínguez M, Hormazábal JC, González G (2011) Characterization of *Vibrio parahaemolyticus* strains isolated in Chile in 2005 and in 2007. J Infect Dev Ctries 7: 502–510. doi: 10.3855/jidc.1228.

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Annex – Supplementary Items

Supplementary Table 1. Oligonucleotide primers and PCR conditions used for detection of the virulence genes of *V. parahaemolyticus*.

Target gene	Primer sequences (5' - 3')	PCR conditions	Product size (bp)	References
toxR	F- GTCTTCTGACGCAATCGTTG R- ATACGAGTGGTTGCTGTCATG	94 °C, 60 s; 63 °C, 90 s; 72 °C, 90 s (30 cycles)	376	[25]
tlh	F-AAAGCGGATTATGCAGAAGCACTG R-GCTACTTTCTAGCATTTTCTCTGC	94 °C, 60 s; 51 °C, 60 s; 72 °C, 60 s (30 cycles)	450	[28]
tdh	F-GTACCGATATTTTGCAAA R- ATGTTGAAGCTGTACTTGA		382	[30]
trh	F- CTCTACTTTGCTTTCAGT R- TACCGTTATATAGGCGCTTA		276	[29]