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Prevalence of *Vibrio parahaemolyticus* in seafood and water environment in the Mekong Delta, Vietnam

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ABSTRACT. *A* total of 449 samples including 385 seafood and 64 water samples in the Mekong Delta of Vietnam collected in 2015 and 2016 were examined. Of 385 seafood samples, 332 (86.2%) samples were contaminated with *Vibrio parahaemolyticus* and 25 (6.5%) samples were pathogenic *V. parahaemolyticus* carrying *tdh* and/or *trh* genes. The *tdh* gene positive *V. parahaemolyticus* strains were detected in 22 (5.7%) samples and *trh* gene positive *V. parahaemolyticus* strains were found in 5 (1.3%) samples. Of 25 pathogenic *V. parahaemolyticus* strains, two strains harbored both *tdh* and *trh* genes and the other 23 strains carried either *tdh* or *trh* gene. Of 64 water samples at aquaculture farms, 50 (78.1%) samples were contaminated with *V. parahaemolyticus*. No *tdh* gene positive *V. parahaemolyticus* strains were detected; meanwhile, *trh* gene positive *V. parahaemolyticus* strain was detected in 1 (1.6%) sample. Twenty-six pathogenic *V. parahaemolyticus* strains isolated were classified into 6 types of O antigen, in which the serotype O3:K6 was detected in 4 strains. All pathogenic strains were group-specific PCR negative except for 4 O3:K6 strains. The result of antimicrobial susceptibility test indicated that pathogenic strains showed high resistance rates to streptomycin (84.6%), ampicillin (57.7%) and sulfisoxazole (57.7%). These findings can be used for understanding microbiological risk of seafood in the Mekong Delta, Vietnam.

KEY WORDS: antimicrobial susceptibility, serotype, *tdh*, *trh*, *Vibrio parahaemolyticus*

Vibrio parahaemolyticus, a gram-negative halophilic bacterium, is a vital agent of food poisoning in human [6]. The infection of this pathogen in human has been reported worldwide [16] and most reported cases link to seafood consumption [7, 18]. Thermostable direct hemolysin (TDH) encoded *tdh* gene and TDH-related hemolysin encoded *trh* gene are considered to be the most important virulent factors of human pathogenic *V. parahaemolyticus* [18]. In Vietnam, the infection of *V. parahaemolyticus* in human has been reported since 1983 [12]. The outbreak of *V. parahaemolyticus* with the predominance of pandemic O3:K6 strain from 1997 to 1999 was reported in Nha Trang in the middle region of Vietnam [4, 24]. Tai *et al*. [22] reported that *V. parahaemolyticus* was isolated at 8.3% from acute diarrheal patients in the South of Vietnam in 2010 and *tdh* or *trh* gene carrying strains dominated 41.7% of these *V. parahaemolyticus* infections. A few information on human pathogenic *V. parahaemolyticus* in environment has been published [22, 23] although human *V. parahaemolyticus* infection has been reported in Vietnam. In this century, a large volume of seafood and seafood products are produced in the Mekong Delta, the South of Vietnam. However, the information on prevalence of *V. parahaemolyticus* in this area has been not fully understood. In an effort to understand the risk of *V. parahaemolyticus* infection in Vietnam, particularly in the Mekong Delta, our objectives were to investigate prevalence of *V. parahaemolyticus* in seafood and water environment in the Mekong Delta and clarify some characteristics of this bacterium such as harboring virulent genes and serotype, harboring pandemic trait and antimicrobial resistance of virulent strains.

MATERIALS AND METHODS

Sample collection

A total of 449 samples including 385 seafood samples and 64 water samples were collected. Of 385 seafood samples, 330

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retail samples including 298 shellfish samples [white hard clam (*Meretrix lyrata*), blood cockle (*Anadara granosa*), mud clam (*Geloina coaxans*), hakf-crenate ark (*Anadara subcrenata*) and antique ark (Anadara antiquata)] *and 32* shrimp samples [banana shrimp (*Penaeus merguiensis*) and greasyback shrimp (*Metapenaeus ensis*)] were purchased from wet markets in Can Tho city and Tra Vinh province in the Mekong Delta in 2015 and 2016 and 55 farming samples including 16 clam samples [white hard clam (*Meretrix lyrata*)] and 39 shrimp samples [white leg shrimp (Litopenaeus vannamei) and black tiger shrimp (*Penaeus monodon*)] were collected at 2 clam farms and 39 shrimp ponds, respectively, in Tra Vinh province in 2016. Of 64 water samples, 22 and 42 samples were collected at 2 clam farms and 42 shrimp ponds, respectively, in Tra Vinh province in 2016. All samples were kept separately in sterile plastic bags placed in polystyrene foam boxes with ice and analyzed immediately as arrival at laboratory.

Enrichment culture and V. parahaemolyticus isolation

For seafood samples, a 25 g portion of seafood was mixed with 225 m*l* of Alkaline Peptone Water (APW, Nissui Co., Ltd., Tokyo, Japan) in sterile stomacher bag to form homogenate solution. For water samples, a 100 m*l* volume of water was mixed well with 100 m*l* of 2 times high concentrate APW. The mixture was incubated at 37°C for 18 hr. After that, a loopful of enrichment culture was inoculated on thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Nissui) and CHROMagar Vibrio (CV) agar (CHROMagar Microbiology, Paris, France) and incubated at 37°C for 18 hr. After incubation, green colonies on TCBS agar and violet colonies on CV agar were picked up and subjected to biochemical tests. The strains showing glucose fermentation without gas production, lysine-positive, mobility-positive, indole-positive, oxidase-positive, VP- negative, no growth without NaCl and growth from 3 to 8% NaCl were identified as *V. parahaemolyticus*. The *V. parahaemolyticus* identified was confirmed by species-specific *toxR* gene detection using PCR method following protocol described by Kim *et al*. [13].

DNA extraction

DNA of *V. parahaemolyticus* strains was extracted using the boiling method as described previously [10].

Pathogenic gene detection

tdh gene: Loop-mediated isothermal amplification assay was applied to detect *tdh* gene using a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan) as described by Yamazaki *et al*. [25]. The incubation was carried out in a Loop realtime tubidimeter (Realoop-30, Eiken Chemical) at 65°C for 60 min, following by 80°C for 2 min. A reaction was considered positive as the turbidity reached 0.1 within 60 min.

trh gene: PCR assay for *trh* gene examination was performed using the protocol described previously [2]. PCR amplified products were checked in 1.5% agarose gels by electrophoresis. After that, the gel was stained with ethidium bromide (0.5 mg/m*l*) and photographed under a UV transilluminator.

Serotyping

V. parahaemolyticus strains harboring *tdh* and/or *trh* genes isolated in this study were serotyped using commercial antisera test kit (Denka Seiken, Tokyo, Japan) following manufacturer's instructions.

Pandemic trait detection

Group-specific PCR (GS-PCR) was carried out to find pandemic strains using protocol described by Matsumoto *et al*. [15].

Antimicrobial resistance examination

The antimicrobial susceptibility test was done using the disk diffusion method following the guideline of Clinical and Laboratory Standard Institute (CLSI) [5]. Nine antimicrobial agents were used in this study including ampicillin (10 μg), chloramphenicol (30 μg), gentamycin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), ofloxacin (10 μg), oxytetracycline (30 μg), streptomycin (10 μg) and sulfisoxazole (250 μg). The density of bacteria was adjusted to reach the turbidity of 0.5 McFarland standard. After that, bacteria were lawn on Muller Hilton agar (Benton, Dickinson and Co., Sparks, MD, U.S.A.) supplemented 3% NaCl plates. Antimicrobial disks (Benton, Dickinson and Co.) were then placed on agar plates and these agar plates were incubated at 37°C for 18 hr.

RESULTS

Prevalence of V. parahaemolyticus in seafood and water samples in the Mekong Delta

Seafood samples: The prevalence of *V. parahaemolyticus* in seafood samples obtained from wet markets and clam and shrimp farms was summarized in Table 1. *V. parahaemolyticus* strains were isolated in 288 of 330 (87.3%) samples from retail shops and in 44 of 55 (80.0%) samples from farms. Of 330 retail seafood samples, *tdh* and/or *trh* gene positive *V. parahaemolyticus* strains were detected in 24 (7.3%) samples. The *tdh* gene positive *V. parahaemolyticus* strains were detected in 22 (6.7%) samples and *trh* gene positive *V. parahaemolyticus* strains were found in 4 (1.2%) samples. Of 24 pathogenic *V. parahaemolyticus* strains, two strains harbored both *tdh* and *trh* genes and the other 22 strains carried either *tdh* or *trh* gene. Regarding to farming seafood samples, none of *tdh* gene positive *V. parahaemolyticus* strains was isolated; meanwhile, *trh* gene positive *V. parahaemolyticus* was found in 1 (1.8%) sample. Of farming seafood samples examined, the *trh* gene positive strain was detected only in the clam sample, dominating 6.3% clam samples collected. No pathogenic *V. parahaemolyticus* strains were detected in shrimp samples

Table 1. Prevalence of *V. parahaemolyticus* in seafood samples in the Mekong Delta, Vietnam

Origin	Samples	No. of samples	No. of *V. parahaemolyticus* positive samples (%)	No. of human pathogenic *V. parahaemolyticus* positive samples (%)		
				tdh gene	*trh* gene	Total
Retail shops	Molluscan shellfish					
	White hard clam	87	79 (90.8)	10 (11.5)	2 (2.3)	11 (12.6)[a)]
	Blood cockle	85	80 (94.1)	5 (5.9)	1 (1.2)	5 (5.9)[a)]
	Mud clam	60	51 (85.0)	4 (6.7)	1 (1.7)	5 (8.3)
	Antique ark	40	32 (80.0)	1 (2.5)	0 (0.0)	1 (2.5)
	Hakf-crenate ark	26	18 (69.2)	2 (7.7)	0 (0.0)	2 (7.7)
	Subtotal	298	260 (87.2)	22 (7.4)	4 (1.3)	24 (8.0)
	Shrimp					
	Banana shrimp	28	25 (89.3)	0 (0.0)	0 (0.0)	0 (0.0)
	Greasyback shrimp	4	3 (75.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Subtotal	32	28 (87.5)	0 (0.0)	0 (0.0)	0 (0.0)
	Subtotal	330	288 (87.3)	22 (6.7)	4 (1.2)	24 (7.3)
Farms	Molluscan shellfish					
	White hard clam	16	16 (100)	0 (0.0)	1 (6.3)	1 (6.3)
	Shrimp					
	White leg shrimp	35	25 (71.4)	0 (0.0)	0 (0.0)	0 (0.0)
	Black tiger shrimp	4	3 (75.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Subtotal	39	28 (71.8)	0 (0.0)	0 (0.0)	0 (0.0)
	Subtotal	55	44 (80.0)	0 (0.0)	1 (1.8)	1 (1.8)
Total		385	332 (86.2)	22 (5.7)	5 (1.3)	25 (6.5)

a) One *V. parahaemolyticus* strain harbored both *tdh* and *trh* genes.

Table 2. Prevalence of *V. parahaemolyticus* in water samples in the Mekong Delta, Vietnam

Water samples	No. of samples	No. of *V. parahaemolyticus* positive samples (%)	No. of human pathogenic *V. parahaemolyticus* positive samples (%)	
			tdh gene	*trh* gene
Molluscan shellfish farms				
White hard clam	22	17 (77.3)	0 (0.0)	1 (4.5)
Shrimp ponds				
White leg shrimp	38	30 (78.9)	0 (0.0)	0 (0.0)
Black tiger shrimp	4	3 (75.0)	0 (0.0)	0 (0.0)
Subtotal	42	33 (78.6)	0 (0.0)	0 (0.0)
Total	64	50 (78.1)	0 (0.0)	1 (1.6)

including in retail shops and in shrimp ponds. All *tdh* and/or *trh* gene carrying strains isolated was *toxR* gene positive (Table 3).

Water samples: Of 64 water samples collected at clam farms and shrimp ponds, 50 (78.1%) samples were *V. parahaemolyticus* positive (Table 2). No *tdh* gene positive *V. parahaemolyticus* was isolated from all samples although only 1 (1.6%) sample harbored *trh* gene positive *V. parahaemolyticus* (Table 2). This *trh* gene positive strain was isolated from clam farming environment, accounting for 4.5% samples collected at this area. The *trh* gene positive strain was also *toxR* gene positive (Table 3).

Serotypes and pandemic trait

The serotypes and pandemic trait of pathogenic *V. parahaemolyticus* strains obtained in this study were shown in Table 3. The pathogenic strains were classified into 6 O serogroups and generated 13 serotypes. Notably, all of 4 O3:K6 strains harboring *tdh* gene from retail shellfish samples showed GS-PCR positive. The pandemic trait was only found in O3:K6 strains, but not in the others.

Antimicrobial resistance

Most of the pathogenic *V. parahaemolyticus* strains isolated in this study showed resistance to streptomycin (84.6%), ampicillin (57.7%) and sulfisoxazole (57.7%) (Table 4). Of 26 pathogenic strains, 5 (19.2%) and 20 (76.9%) strains showed resistance against one and more than one antimicrobial agents, respectively; while only 1 strain showed no resistance to antimicrobial agents (Table 5).

Table 3. Characteristics of human pathogenic *V. parahaemolyticus* strains isolated from seafood and water samples in the Mekong Delta, Vietnam

Serotype	Origin	*tdh* gene	*trh* gene	*toxR* gene	GS-PCR
O1:K1	Retail shellfish	-	+	+	-
O1:K32	Retail shellfish	+	-	+	-
O1:K_{UT}[a)]	Retail shellfish	+	+	+	-
O1:K_{UT}	Retail shellfish	+	+	+	-
O1:K_{UT}	Retail shellfish	+	-	+	-
O2:K_{UT}	Retail shellfish	+	-	+	-
O2:K_{UT}	Retail shellfish	+	-	+	-
O2:K_{UT}	Retail shellfish	+	-	+	-
O3:K6	Retail shellfish	+	-	+	+
O3:K6	Retail shellfish	+	-	+	+
O3:K6	Retail shellfish	+	-	+	+
O3:K6	Retail shellfish	+	-	+	+
O3:K7	Retail shellfish	+	-	+	-
O3:K7	Retail shellfish	+	-	+	-
O3:K_{UT}	Retail shellfish	+	-	+	-
O3:K_{UT}	Clam at clam farm	-	+	+	-
O4:K29	Retail shellfish	+	-	+	-
O4:K34	Retail shellfish	+	-	+	-
O4:K42	Retail shellfish	+	-	+	-
O4:K42	Retail shellfish	+	-	+	-
O4:K42	Retail shellfish	+	-	+	-
O4:K_{UT}	Retail shellfish	+	-	+	-
O4:K_{UT}	Retail shellfish	+	-	+	-
O4:K_{UT}	Retail shellfish	+	-	+	-
O5:K47	Retail shellfish	-	+	+	-
O8:K_{UT}	Water at clam farm	-	+	+	-

a) Untypeable.

Table 4. Antimicrobial resistance of human pathogenic *V. parahaemolyticus* (n=26)

Antimicrobial agents	No. of resistant strains (%)
Streptomycin	22 (84.6)
Ampicillin	15 (57.7)
Sulfisoxazole	15 (57.7)
Kanamycin	2 (7.7)
Chloramphenicol	0 (0.0)
Gentamycin	0 (0.0)
Nalidixic acid	0 (0.0)
Oxfloxacin	0 (0.0)
Oxytetracycline	0 (0.0)

Table 5. Resistant patterns of human pathogenic *V. parahaemolyticus* strains against 9 antimicrobial agents

Resistant patterns	No. of strains
KM-AMP-SFX-STM[a)]	1
KM-AMP-STM	1
AMP-SFX-STM	6
SFX-STM	6
AMP- STM	4
AMP-SFX	2
STM	4
AMP	1
No resistance	1
Total	26

a) KM: Kanamycin; AMP: Ampicillin; SFX: Sulfisoxazole; STM: Streptomycin.

DISCUSSIONS

The pathogenicity of *V. parahaemolyticus* causing foodborne illness in human is usually associated with *tdh* and *trh* genes [18]. The contamination of this pathogen has been reported in seafood samples in some Southeast Asian countries such as Thailand [3, 17], Malaysia [2, 17, 19] and Indonesia [17]. However, few information on the prevalence of this pathogen in environment in the Mekong Delta has been reported. In this study, *tdh* and *trh* gene positive *V. parahaemolyticus* was isolated from retail shellfish and shellfish farms. This is the first report on the detection of human pathogenic *V. parahaemolyticus* in food in the Mekong Delta, Vietnam.

The human pathogenic *V. parahaemolyticus* is usually detected relatively at a high rate in molluscan shellfish samples. The *tdh* gene positive *V. parahaemolyticus* was detected from molluscan shelfish at 12% in Thailand [3], 11.1% in Malaysia and 9.1% in Indonesia [17]. It is known that molluscan shellfish are filter feeders and can accumulate pathogenic *V. parahaemolyticus* in their guts, providing high prevalence of this pathogenic bacterium compared to other species such as shrimp and fish [17, 20]. Therefore, the prevalence rate of pathogenic *V. parahaemolyticus* in shellfish samples seems to be higher than that in shrimp in this study. Moreover, many of retail shops in this study were located near the coast in the Mekong Delta. Shellfish from those shops were usually sold immediately after they were harvested at the coast. Therefore, prevalence of pathogenic *V. parahaemolyticus* in retail shellfish seems to reflect that in the environment in this coastal area in this study although *tdh* gene positive *V. parahaemolyticus* was not detected from farming shellfish and environment samples.

Serotype O3:K6 is known to be the predominant serotype isolated from *V. parahaemolyticus* infection in human [16]. The GS-PCR positive O3:K6 strain is indicated as the pandemic strain [15]. In this study, all of 4 O3:K6 strains detected in retail shellfish samples showed GS-PCR positive. These results indicate that the pandemic *V. parahaemolyticus* strain is prevalent in the environment in the Mekong Delta. Therefore, we should pay more attention on human *V. parahaemolyticus* infection in this region.

Pathogenic *V. parahaemolyticus* strains isolated in this study showed high resistance rates to streptomycin, ampicillin and sulfisoxazole. Similar observations were reported elsewhere in Southeast Asian countries [1, 9, 14, 21]. However, Marlina *et al*. [14] reported human pathogenic *V. parahaemolyticus* originated from Indonesia showed high resistance rates to tetracycline and gentamycin, although our isolates showed no resistance to those antibiotics. Almost all pathogenic *V. parahaemolyticus* strains examined in this study demonstrated multiple antimicrobial resistances. Other studies in some Southeast Asian countries also

recorded similar results [1, 14]. In fact, several antimicrobial agents such as streptomycin, ampicillin and sulfisoxazone have been frequently used in agriculture and aquaculture for the last some decades; therefore, the resistance of bacteria to those antimicrobial agents could be facilitated by selective pressure [8, 11].

In conclusion, human pathogenic *V. parahaemolyticus* is prevalent in seafood relatively at high rate in the Mekong Delta and the pandemic serotype O3:K6 distributed in this area. Furthermore, pathogenic *V. parahaemolyticus* isolated in this area showed resistance to several antimicrobial agents and performed multidrug resistance. These findings can be used for monitoring microbiological risk of seafood in the Mekong Delta.

CONFLICT OF INTEREST. No conflicts were declared.

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