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Serogroup, virulence, and molecular traits of *Vibrio parahaemolyticus* isolated from clinical and cockle sources in northeastern Thailand

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ABSTRACT

Vibrio parahaemolyticus is responsible for seafood-borne gastroenteritis worldwide. Isolates of *V. parahaemolyticus* from clinical samples (n = 74) and cockles (*Anadara granosa*) (n = 74) in Thailand were analyzed by serotyping, determination of virulence and related marker genes present, response to antimicrobial agents, and genetic relatedness. Serological analysis revealed 31 different serotypes, 10 of which occurred among both clinical and cockle samples. The clinical isolates commonly included the pandemic serogroup O3:K6, while a few of the cockle isolates exhibited likely pandemic serovariants such as O3:KUT and O4:KUT, but not O3:K6. The pandemic (*orf8* gene-positive) strains were more frequently found among clinical isolates (78.4%) than cockle isolates (28.4%) ($p < 0.001$). Likewise, the virulence and related marker genes were more commonly detected among clinical than cockle isolates; i.e., *tdh* gene (93.2% versus 29.7%), *vcrD2* (97.3% versus 23.0%), *vopB2* (89.2% versus 13.5%), *vopT* (98.6% versus 36.5%) (all $p < 0.001$) and *trh* (10.8% versus 1.4%) ($p < 0.05$). Pulsed-field gel electrophoresis of *Not*I-digested genomic DNA of 41 randomly selected *V. parahaemolyticus* isolates representing different serotypes produced 33 pulsotypes that formed 5 different clusters (clonal complexes) (A-E) in a dendrogram. *Vibrio parahaemolyticus* O3:K6 and likely related pandemic serotypes were especially common among the numerous clinical isolates in cluster C, suggesting a close clonal link among many of these isolates. Most clinical and cockle isolates were resistant to ampicillin. This study indicates that O3:K6 and its likely serovariants based on the PFGE clusters, are causative agents. Seafoods such as cockles potentially serve as a source of virulent *V. parahaemolyticus*, but further work is required to identify possible additional sources.

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1. Introduction

Vibrio parahaemolyticus is a Gram-negative halophilic bacterium found in marine and estuarine environments (Alam et al., 2009; Meador et al., 2007). This organism is a major causative agent of seafood-borne gastroenteritis worldwide and is acquired by consumption of raw or undercooked seafood, especially shellfish (Meador et al., 2007; Su and Liu, 2007). Although the gastroenteritis caused by *V. parahaemolyticus* is not severe, the infection can cause septicemia in people with underlying conditions, such as liver disease or immune disorders (Su and Liu, 2007).

V. parahaemolyticus can be classified into 13 O serotypes and 71 K serotypes based on somatic (O) antigens and capsular (K) antigens (Chen et al., 2012; Han et al., 2008; Nair et al., 2007). Since 1996, serotype O3:K6 and its serovariants (O4:K68, O1:K25 and O1:KUT) have been responsible for most outbreaks worldwide including Thailand (Chowdhury et al., 2000; Laohaprertthisan et al., 2003). Currently, 22 serotypes are regarded as variants of O3:K6 and are collectively referred to as pandemic strains (Nair et al., 2007; Tsai et al., 2013).

The pathogenesis of *V. parahaemolyticus* depends on the presence of virulence factors including thermolabile hemolysin (TLH), thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) (Letchumanan et al., 2014). Other major virulence factors in this and other *Vibrio* species include the type 3 secretion systems (T3SSs), which deliver bacterial effectors into host cytoplasm, leading to cell lysis caused by a multifaceted host cell infection process (Burdette et al., 2008; Ceccarelli et al., 2013; Noriea et al., 2010). There are two sets of T3SSs, T3SS1 and T3SS2. T3SS1 is found in all *V. parahaemolyticus* strains (Liu and Chen, 2013; Park et al., 2004) while T3SS2 is present only in Kanagawa phenomenon-positive strains (Kodama et al., 2007; Park et al., 2004). T3SS1 and T3SS2

Abbreviations: V. parahaemolyticus, *Vibrio parahaemolyticus*; PFGE, pulsed-field gel electrophoresis; PCR, polymerase chain reaction.

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are involved in cytotoxicity and cytotoxicity/enterotoxicity in eukaryotic cells, respectively (Hiyoshi et al., 2010). T3SS2, which includes two lineages, T3SS2α and T3SS2β, may play a role in the survival of environmental strains (Caburlotto et al., 2010; Matz et al., 2011). In T3SS1, important genes are *vcrD1*, *vopA*, *vopS* and *vpa450* whereas T3SS2α genes include *vcrD2*, *vopB2* and *vopT* (Kodama et al., 2007; Ono et al., 2006; Park et al., 2004; Tsai et al., 2013). The *vcrD1* and *vcrD2* genes encode essential inner-membrane components including *Vibrio* calcium-response protein (VcrD) (Park et al., 2004; Rattanama et al., 2009; Tsai et al., 2013). The *vopB2* gene encodes a translocation protein involved in the contact-dependent activity of pore formation in infected cells while the *vopT* gene encodes an effector protein that is a homolog of the *Pseudomonas* exoenzymes T and S (Kodama et al., 2007). Molecular identification of the T3SS2α genes present is one method for establishing the pathogenicity of *V. parahaemolyticus* isolates (Noriea et al., 2010; Tsai et al., 2013). The *orf8* gene, which encodes ORF8, an adhesive protein of the filamentous phage f237, is another useful marker for pandemic O3:K6 strains (Myers et al., 2003; Nasu et al., 2000).

In Thailand, contaminated seafoods are a major source of food-borne pathogens such as *V. parahaemolyticus*. Cockle (*Anadara granosa*) is a marine bivalve known to harbor microorganisms (Bilung et al., 2005). Most cockles are grown in the south of Thailand, especially Surat Thani (Jarernpornnipat et al., 2003), and are transported to other provinces or to neighboring countries such as Lao People's Democratic Republic (Lao PDR). Khon Kaen municipality is a large city in the center of northeastern Thailand and close to Nong Khai, the gateway to the Lao capital, Vientiane. The present study was designed to determine serogroup, virulence, and molecular characteristics of naturally occurring *V. parahaemolyticus* strains isolated from cockle samples, and their phenotypic and genetic relationships with *V. parahaemolyticus* strains associated with diarrhea cases in northeastern Thailand.

2. Materials and methods

2.1. Sources of V. parahaemolyticus isolates

A total of 148 *V. parahaemolyticus* isolates used in this study included 74 from clinical sources and 74 from cockles (*A. granosa*), obtained between March, 2010 and January, 2012. The clinical isolates were collected from rectal swab samples of patients with diarrhea in Khon Kaen Province of northeastern Thailand. Seventy-four *V. parahaemolyticus* isolates were obtained from 143 cockle samples collected from three cockle farms in Surat Thani (Southern Thailand) and also from markets in Nong Khai (Northeast Thailand bordering Lao PDR) and Khon Kaen (Fig. S1). *V. parahaemolyticus* ATCC17802 was used as the reference strain in the present study. This study was approved by the Institutional Human Ethics Committee (HE 551043).

2.2. Isolation and identification of V. parahaemolyticus isolates

The seventy-four cockle isolates of *V. parahaemolyticus* were those reported by Senachai et al. (2013). In brief, 250 g of each cockle sample was cut into small pieces and suspended in 250 ml of phosphate-buffered saline (PBS). A 20 ml (10 g) aliquot of cockle suspension was subsequently added to 80 ml of alkaline peptone water (10X APW; Oxoid, England) and incubated at 37 °C for 6 h. A 5 μl aliquot was streaked onto thiosulfate-citrate-bile salt-sucrose (TCBS) agar (Eiken, Japan) and incubated at 37 °C for 18 h. Suspected *V. parahaemolyticus* colonies from TCBS agar were identified using standard biochemical tests as reported by Ramamurthy and Nair (2007).

The clinical isolates of *V. parahaemolyticus* (n = 74) included in the present study were obtained from rectal swab samples from patients with diarrhea by culturing overnight on TCBS agar and confirmed using the same procedure as the cockle isolates (Ramamurthy and Nair, 2007).

2.3. Serotyping

All *V. parahaemolyticus* isolates were serotyped using commercial antisera (Denka Seiken, Tokyo, Japan) following the methods at the Reference Laboratory of the National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand.

2.4. Genomic DNA extraction

DNA from *V. parahaemolyticus* was extracted employing the method of Bilung et al. (2005) with slight modification. Briefly, a 200 μl aliquot of overnight bacterial culture was centrifuged at 14,000 *g* for 5 min. The pellet was re-suspended in sterile distilled water and was boiled for 10 min. The tube was placed on ice for 10 min and then centrifuged at 2000 *g* for 5 min. The supernatant was used as the DNA template for PCR assays.

2.5. PCR assays

The *tlh*, *tdh*, *trh*, *vcrD1*, *vcrD2*, *vopB2*, *vopT* and *orf8* genes were detected using optimized uniplex PCR assays. The oligonucleotide primers used in this study are listed in Table 1. Amplification reactions were conducted in a total volume of 25 μl containing 2.5 μl of 1X PCR buffer, 0.2 mM of deoxynucleotide triphosphate (dNTPs), 0.3 μM of each primer, 0.5 U of *Taq* DNA polymerase and 300 ng of genomic DNA (2 μl). PCR assay was performed using a Bio-Rad C1000 thermal cycler. The amplified DNA was analyzed by 1.5% agarose-gel electrophoresis and visualized under UV light (Bio-Rad Gel™ Doc XR+ Imager) after ethidium bromide staining.

2.6. Pulsed-field gel electrophoresis (PFGE)

PFGE for the investigation of the relationship between various isolates of *V. parahaemolyticus* was performed according to the Rapid Standardized Laboratory Protocol for Molecular Subtyping of *V. parahaemolyticus* (Centers for Disease Control and Prevention, 2009), with some modifications. Briefly, each *V. parahaemolyticus* isolate was grown on tryptic soy agar (TSA) supplemented with 2% NaCl. Chromosomal DNA of each isolate was digested with 40 U of *Not*I restriction enzyme (Promega, Southampton, United Kingdom). DNA from *Salmonella* serotype Braenderup H9812, digested with 50 U of *Xba*I, was used as a molecular size marker. PFGE was performed in 1% SeaKem Gold agarose (FMC, Lonza, Rockland, ME) in 0.5X Tris borate EDTA buffer with 50 μM thiourea using a CHEF DRIII system (Bio-Rad, Hercules, Calif.) at 6.0 V/cm (Banerjee and Farber, 2009). Pulse times were ramped from 2 to 35 s during an 18-h run at 6.0 V/cm.

2.7. Statistical analysis

Correlations between the bacterial sources and the virulence gene contents were analyzed using a Chi-square test or Fisher's exact test. *P*-values < 0.05 were considered statistically significant. The PFGE patterns were analyzed using BioNumeric software (version 4.6). Genetic relationships were determined based on the Dice similarity coefficients and dendrograms created using UPGMA (the unweighted pair group method with arithmetic averages).

2.8. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of *V. parahaemolyticus* isolates was performed using the disk diffusion assay method according to the Clinical and Laboratory Standards Institute (CLSI) (Clinical and

Table 1
Oligonucleotide primers and PCR conditions used for detection of the species-specific gene (*tlh*) and virulence-associated genes of *V. parahaemolyticus*.

Genes	Amplicon sizes (bp)	Primer sequences	PCR conditions	References
tlh	170	F-5′- GCGGATTATGCAGAAGCACTG -3' R-5′- TGTGCCTTGATGAACTCGTTC -3'	94 °C, 30 s; 58 °C, 30 s; 72 °C, 45 s (35 cycles)	Modified from Rizvi and Bej (2010)
tdh	269	F-5′- GTAAAGGTCTCTGACTTTTGGAC -3' R-5′- TGGAATAGAACCTTCATCTTCACC -3'		Bej et al. (1999)
trh	499	F-5′- TTGGCTTCGATATTTTCAGTATCT -3' R-5′- ATAACAAACATATGCCCATTTCCG -3'		Modified from Bej et al. (1999)
orf8	369	F-5′- AGGACGCAGTTACGCTTGATG -3' R-5′- CTAACGCATTGTCCCTTTGTAG -3′		Myers et al. (2003)
vcrD1	493	F-5′- CTGCTGGTCTTGTTCGCTCT -3′ R-5′- TCTGGTCGCTTCCTTCTGTG -3′	94 °C, 1 min; 53 °C, 1 min; 72 °C, 1 min (35 cycles)	Tsai et al. (2013)
vcrD2	300	F-5′- GTTGGTGCTCGCTTCTCTCT -3′ R-5′- CCCATCCCCTACTGTCAAGA -3′		Tsai et al. (2013)
vopB2	527	F-5′- GGGGGCAAGCTAATAAAGAGAT -3′ R-5′- GTTAAAGCTGAGCAACATCGTG -3′		Tsai et al. (2013)
vopT	690	F-5′- GTGAAGGTTTGTAGAATACATACGGAAA -3′ R-5′- TCACTTAGCTAAATCTAGCGCATC -3'		Kodama et al. (2007)

Laboratory Standards Institute, 2009). The commercial disks (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England) included ampicillin (10 μg), cefotaxime (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), nalidixic acid (30 μg), norfloxacin (10 μg), ofloxacin (5 μg), tetracycline (30 μg) and trimethoprim-sulfamethoxazole (1.25/23.75 μg). *Escherichia coli* ATCC 25922 was used for quality control. After incubation at 37 °C for 18 h, the diameters of inhibition zones were measured and interpreted according to the CLSI requirements.

3. Results

3.1. Serotyping of V. parahaemolyticus isolates

Thirty-one different serotypes were identified from among the 148 clinical and cockle isolates of *V. parahaemolyticus* (Fig. 1), 17 from clinical and 24 from cockle isolates. Seven serotypes, including the pandemic serovariants such as O1:K25, O1:KUT and O3:KUT, were found among both clinical and cockle isolates. The pandemic serotypes O3:K6 and O4:K68 were found only in clinical isolates and the first of these was the most frequently detected serotype among the clinical isolates. The pandemic serotypes O3:KUT and O4:KUT were the most frequent serotypes among the cockle isolates (Fig. 1).

3.2. Distribution of virulence-associated genes in clinical and cockle isolates of V. parahaemolyticus

The *tlh* gene was amplified from all 148 isolates by PCR, confirming them to be *V. parahaemolyticus*. The T3SS1 gene, *vcrD1* was also found in all isolates. Virulence (*tdh* and *trh*) and related T3SS2α genes (*vcrD2*, *vopB2*, and *vopT*) were significantly more frequently detected in the clinical isolates than in the cockle isolates ($p < 0.001$; except *trh*, $p < 0.05$) (Table 2). The tdh^+ trh^- combination was the most commonly found among clinical isolates, at a significantly higher frequency than among cockle isolates. The *orf8* gene was detected in 78.4% of the clinical isolates and 28.4% of the cockle isolates and was found more frequently in the *tdh*-positive pandemic serotypes predominantly associated with diarrhea (Table 2). T3SS2α, *tdh*, and *orf*8 genes and pandemic serotypes were significantly more frequently found among the clinical isolates relative to those from cockles (Table 2).

3.3. PFGE patterns of V. parahaemolyticus clinical and cockle isolates and relationship with virulence gene profiles

Forty-one randomly selected *V. parahaemolyticus* isolates (24 clinical isolates, 16 cockle isolates, and one reference strain) belonging to different serotypes were subjected to molecular fingerprinting analysis by PFGE. *V. parahaemolyticus* strains from Thailand proved to be very diverse genetically as 33 different PFGE patterns (pulsotypes) were obtained from 41 isolates. Cluster analysis of the PFGE images separated the 33 pulsotypes into five distinct clusters (A-E); and on the basis of their virulence-associated gene profiles, 13 different groups were also identified (Fig. 2). Half of the clinical isolates (12/24), all of which were positive for *tdh*, belonged to cluster C ($p = 0.015$) (Fig. 2). Most of the clinical isolates belonged to group 2 (14/24 isolates, 58.3%) ($p < 0.0001$), which included mainly the pandemic serotype O3:K6 and its serovariants, O4:K68, O1:K25, O3:KUT and O1:KUT (Fig. 2). *V. parahaemolyticus* strains of cockle origin shared clusters (A, B, and E) with those of clinical origin, which included closely related pandemic serogroup strains and the pandemic serovariants, but did not appear clonally related to clinical isolates in those clusters.

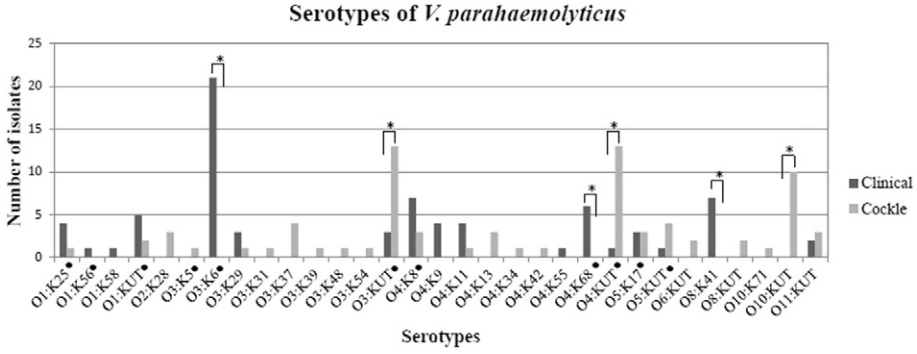


Fig. 1. Distribution of serotypes among clinical and cockle isolates of *V. parahaemolyticus*. Dots (.) represent the pandemic strains. Stars (*) indicate significant differences (p-value < 0.05) in serotype frequency between clinical isolates and cockle isolates.

Table 2
Distribution of virulence-associated genes in clinical and cockle isolates of *V. parahaemolyticus*.

Genes	Number of isolate (%)		*P*-values
	Clinical (n = 74)	Cockle (n = 74)	
Toxin genes			
tlh	74 (100)	74 (100)	1.000
tdh	69 (93.2)	22 (29.7)	<0.0001
trh	8 (10.8)	1 (1.4)	0.017
Combinations of toxin genes			
tdh$^+$, *trh*$^+$	6 (8.1)	1 (1.4)	0.058
tdh$^+$, *trh*$^-$	63 (85.1)	21 (28.4)	<0.0001
tdh$^-$, *trh*$^+$	2 (2.7)	0 (0.0)	0.248
tdh$^-$, *trh*$^-$	3 (4.1)	52 (70.3)	<0.0001
Total	74	74	
T3SS1 gene			
vcrD1	74 (100)	74 (100)	1.000
T3SS2α genes			
vcrD2	72 (97.3)	17 (23.0)	<0.0001
vopB2	66 (89.2)	10 (13.5)	<0.0001
vopT	73 (98.6)	27 (36.5)	<0.0001
Combinations of T3SS2α genes			
vcrD2 $^+$, *vopB2* $^+$, *vopT* $^+$	65 (87.8)	6 (8.1)	<0.0001
vcrD2 $^+$, *vopT* $^+$	7 (9.5)	5 (6.8)	0.547
vopB2 $^+$, *vopT* $^+$	1 (1.4)	1 (1.4)	1.000
vcrD2$^+$	0 (0.0)	6 (8.1)	<0.0001
vopB2$^+$	0 (0.0)	3 (4.1)	0.122
vopT$^+$	0 (0.0)	15 (20.1)	<0.0001
None	1 (1.4)	38 (51.4)	<0.0001
Total	74	74	
Pandemic strains			
Pandemic serotype	52 (70.3)	39 (52.7)	0.028
orf8$^+$	58 (78.4)	21 (28.4)	<0.0001
Pandemic serotype, *orf8* $^+$	45 (60.8)	14 (18.9)	<0.0001
orf8 $^+$, *tdh* $^+$	55 (74.3)	5 (6.8)	<0.0001

Many *V. parahaemolyticus* strains possessing secretory system related genes *vcrD2*, *vopB2* and *vopT* and the pandemic marker gene *orf8* (i.e. group 2) were related closely as they belonged to cluster C ($p = 0.002$) (Fig. 2).

3.4. Antimicrobial susceptibility assay of *V. parahaemolyticus* clinical and cockle isolates

Vibrio parahaemolyticus isolates from clinical (n = 74) and cockle (n = 74) samples were tested with 10 antimicrobial agents (Table S1). Most of the clinical (72/74, 97.3%) and cockle (62/74, 83.8%) isolates were resistant to ampicillin. A few of the clinical isolates also showed resistance to cefotaxime (1/74, 1.4%), gentamicin (2/74, 2.7%), norfloxacin (3/74, 4.1%), ofloxacin (2/74, 2.7%), tetracycline (3/74, 4.1%) and trimethoprim-sulfamethoxazole (3/74, 4.1%). Cockle isolates were susceptible to all of these antimicrobial agents.

4. Discussion

In Thailand, *V. parahaemolyticus* is often found associated with gastroenteritis cases, although the source of the infection remains unclear. Here, we show that both clinical and cockle isolates of *V. parahaemolyticus* in Thailand are genetically highly diverse and those from cockle (*A. granosa*), a common seafood item in Thailand, have the potential to be pathogenic.

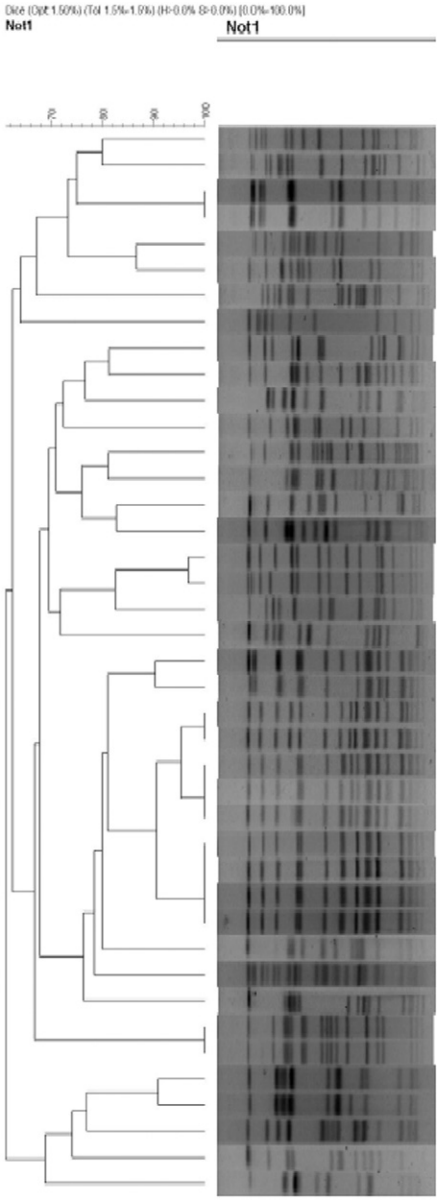
In this study, the pandemic serotype O3:K6 was the one most commonly associated with *V. parahaemolyticus* diarrhea, as reported earlier from Thailand (Laohaprertthisan et al., 2003). Most of the clinical isolates (70%) and many of the cockle isolates (53%) belonged to the pandemic serogroup. With respect to the combination of pandemic marker *orf8* and pandemic serotype, this finding confirms that the *orf8* gene is useful specific marker of pandemic strains.

Serotype O3:K6 emerged as a major cause of diarrhea in India and rapidly spread worldwide, causing massive diarrheal outbreaks (Ansaruzzaman et al., 2005; DePaola et al., 2000; Nair et al., 2007). Subsequent studies in India and southern Thailand showed that additional serotypes such as O4:K68, O1:K25, O1:K41, and O1:KUT carried marker genes present in the pandemic pathogen O3:K6 (Chowdhury et al., 2000; Laohaprertthisan et al., 2003; Nair et al., 2007). In the present study, the pandemic serotypes O3:K6 and O4:K68 were found among the clinical isolates but not among the cockle isolates, suggesting that clinical cases may not be the direct result of eating cockles. However, other pandemic serotypes such as O3:KUT, O4:KUT, O1:K25, O1:KUT, and O4:K8 did occur in cockles. The high sero-diversity of *V. parahaemolyticus* observed among the cockle and clinical isolates in the present study is consistent with the results reported earlier from Thailand (Laohaprertthisan et al., 2003) and provides evidence for a high frequency of horizontal gene transfer or recombination in *V. parahaemolyticus* in the region.

The virulence-associated gene profiles of clinical (pathogenic) and of environmental *V. parahaemolyticus* strains can vary greatly among geographic regions (Alam et al., 2009; Tsai et al., 2013). In the present study, the majority of clinical isolates had the *tdh* gene, but not *trh* and a few harbored *trh*, but not *tdh*, as also observed in Taiwan (Tsai et al., 2013) and in China (Vongxay et al., 2008). We also found that clinical isolates harboring the *tdh* gene were significantly more likely to also harbor T3SS2α genes, in agreement with some previous studies (Noriea et al., 2010; Tsai et al., 2013). In contrast, *V. parahaemolyticus* strains responsible for diarrhea in North America and Canada frequently exhibited the *tdh*$^+$ *trh*$^+$ genotype (Banerjee et al., 2014; Jones et al., 2012), which is uncommon in Thailand. Environmental isolates around the world vary considerably in frequencies of these two genes. In this study, the *tdh*$^+$ *trh*$^-$ genotype was found in 28.4% of cockle isolates, and *tdh*$^-$ *trh*$^-$ in 70.3%. In contrast, in North America, the *trh* gene was slightly more frequent among isolates from oysters (Jones et al., 2012) and in Malaysia, isolates from cockles more frequently harbored the *trh* gene than the *tdh* gene (Bilung et al., 2005). In some studies, however, very few or no environmental isolates carried either gene (Ceccarelli et al., 2013; Tsai et al., 2013; Vongxay et al., 2008).

The observed high frequency of virulence-associated genes among the clinical isolates responsible for diarrhea in Thailand supports the supposition that recombination events can create pathogenic strains. Although virulence-associated genes were found to be highly dispersed among the cockle isolates in the present study, a complex natural selection process could generate virulent strains in the environment (Alam et al., 2009; Tsai et al., 2013; Turner et al., 2013). It is also plausible that human hosts, "selecting" environmental strains based on the presence of necessary virulence-associated genes co-occurring in the human gut, could drive the emergence of a more virulent strain (Theethakaew et al., 2013). The human upper intestine is proposed to be a particularly suitable niche for the intra- and inter-specific horizontal transfer of genetic material that is important in enhancing pathogenicity (Haley et al., 2010; Larocque et al., 2005; Okada et al., 2009; Wang et al., 2011).

In Thailand, *V. parahaemolyticus* has been the most frequent cause of food poisoning and is responsible for approximately 44% of all food poisoning cases with the highest incidence occurring in the northeastern region (Bureau of Epidemiology, T., 2013). *V. parahaemolyticus* is generally most prevalent during the warm summer season (Yeung and Boor, 2004). In Thailand, the peak of food poisoning cases occurs towards the end of summer (the rainy season, May – July), especially when temperatures reach approximately 30 °C (Hinjoy et al., 2014). An increase in temperature by 1 °C was correlated with an increase of 15% in food poisoning cases in the following month (Hinjoy et al., 2014). The role of other environmental factors (pH, oxygen, nutrients etc.) and of cultural factors (e.g. eating and cooking practices, sanitation and hygiene) may be involved in the epidemiology of *V. parahaemolyticus*. However, the interplay of all these factors and pandemic serotypes such as O3:K6, is not understood. The incidence of infection by the pandemic strains did not increase between 2006 and 2010 in southern Thailand (Thongjun et al., 2013). During that period, most of the isolates (51.3–73.6%)



Strain	Source	Place	Serotype	Group of virulence gene profiles: group	tdh	trh	vcrD2	vopB2	vopT	orf8	Month, Year	
Vp93	Cockle	Surat Thani 2	O4:K11	13	-	-	-	-	-	-	Sep, 2010	A
Vp79	Cockle	Surat Thani 1	O10:KUT	8	-	-	-	-	+	+	Mar, 2010	
Vp5	Clinical	Khon Kaen 1	O3:K29	3	+	-	+	+	+	-	Feb, 2011	
Vp1	Clinical	Khon Kaen 1	O3:K29	2	+	-	+	+	+	+	Dec, 2010	
Vp80	Cockle	Surat Thani 2	O1:KUT*	9	-	-	-	-	-	+	Mar, 2010	
Vp85	Cockle	Surat Thani 1	O3:KUT*	9	-	-	-	-	-	+	Jun, 2010	
Vp119	Cockle	Nong Khai 4	O5:KUT*	7	+	-	-	-	-	-	Apr, 2010	
Vp37	Clinical	Khon Kaen2	O11:KUT	4	+	-	-	+	+	-	Jul, 2011	
Vp130	Cockle	Khon Kaen 3	O1:K25*	8	-	-	-	-	+	+	May, 2010	B
Vp132	Cockle	Khon Kaen 1	O4:KUT*	4	+	-	-	+	+	-	May, 2010	
Vp123	Cockle	Nong Khai 3	O10:KUT	6	+	-	+	-	+	-	Apr, 2010	
Vp86	Cockle	Surat Thani 1	O4:KUT*	9	-	-	-	-	-	+	Jul, 2010	
Vp107	Cockle	Nong Khai 4	O4:K8*	7	+	-	-	-	-	-	Apr, 2010	
Vp41	Clinical	Khon Kaen 1	O1:KUT*	5	+	+	+	-	+	-	Jul, 2011	
Ref.	Clinical	Japan	O1:K1	-	-	-	-	-	-	-	1965	
Vp22	Clinical	Khon Kaen 1	O4:KUT*	12	-	-	+	-	+	+	May, 2011	
Vp9	Clinical	Khon Kaen 1	O5:K17*	10	-	+	+	-	+	-	Mar, 2011	
Vp70	Clinical	Khon Kaen 1	O5:K17*	1	+	+	+	+	+	+	Oct, 2011	
Vp101	Cockle	Nong Khai 1	O1:KUT*	7	+	-	-	-	-	-	Apr, 2010	
Vp116	Cockle	Nong Khai 2	O3:KUT*	7	+	-	-	-	-	-	Apr, 2010	
Vp63	Clinical	Khon Kaen 2	O1:K25*	2	+	-	+	+	+	+	Aug, 2011	C
Vp4	Clinical	Khon Kaen 1	O1:K25*	2	+	-	+	+	+	+	Feb, 2011	
Vp15	Clinical	Khon Kaen 1	O4:K68*	2	+	-	+	+	+	+	Apr, 2011	
Vp25	Clinical	Khon Kaen 1	O4:K68*	2	+	-	+	+	+	+	Jun, 2011	
Vp66	Clinical	Khon Kaen 2	O3:K6*	1	+	+	+	+	+	+	Oct, 2011	
Vp21	Clinical	Khon Kaen 1	O1:KUT*	2	+	-	+	+	+	+	June, 2011	
Vp74	Clinical	Khon Kaen 2	O3:K6*	2	+	-	+	+	+	+	Jan, 2012	
Vp12	Clinical	Khon Kaen 1	O3:K6*	2	+	-	+	+	+	+	Apr, 2011	
Vp10	Clinical	Khon Kaen 1	O3:K6*	2	+	-	+	+	+	+	Feb, 2011	
Vp14	Clinical	Khon Kaen 2	O3:KUT*	2	+	-	+	+	+	+	May, 2011	
Vp24	Clinical	Khon Kaen 1	O3:KUT*	2	+	-	+	+	+	+	July, 2011	
Vp89	Cockle	Surat Thani 2	O5:K17*	11	-	-	+	+	+	+	Aug, 2010	
Vp67	Clinical	Khon Kaen 1	O4:K11	1	+	+	+	+	+	+	Sep, 2011	
Vp138	Cockle	Khon Kaen 2	O5:KUT*	11	-	-	+	+	+	+	May, 2010	
Vp50	Clinical	Khon Kaen 1	O8:K41	3	+	-	+	+	+	-	Aug, 2011	D
Vp71	Clinical	Khon Kaen 1	O8:K41	2	+	-	+	+	+	+	Oct, 2011	
Vp35	Clinical	Khon Kaen 2	O4:K8*	2	+	-	+	+	+	+	Jul, 2011	E
Vp46	Clinical	Khon Kaen 1	O4:K8*	1	+	+	+	+	+	+	Aug, 2011	
Vp54	Clinical	Khon Kaen 1	O4:K11	2	+	-	+	+	+	+	Sep, 2011	
Vp98	Cockle	Surat Thani 3	O10:KUT	9	-	-	-	-	-	+	Sep, 2010	
Vp135	Cockle	Khon Kaen 1	O11:KUT	11	-	-	+	+	+	+	Apr, 2010	

Fig. 2. PFGE-*Not*I restriction patterns and clusters of the clinical and cockle isolates of *V. parahaemolyticus*. The source, place, serotype, group of genes, virulence gene profile and PFGE type are listed on the right of each strain. Stars (*) indicate pandemic serotype strains. The number following each place represents the market in that province. The dendrogram was produced using Bionumeric software.

were tdh^+ trh^- and group-specific PCR-positive pandemic strains (Thongjun et al., 2013). In our study, clinical samples were collected in northeastern Thailand during 2010–2012 and isolates of *V. parahaemolyticus* from these frequently belonged to the pandemic serogroup O3:K6 strains. However, this pandemic serogroup was not directly found among isolates from cockle samples from the same region. In addition to possible recombination events, there are several possible reasons for this discrepancy. Clinical samples may not have been collected at the same time point as cockle samples. Most of the clinical isolates were collected in Khon Kaen, and not from Nong Khai or Surat Thani, where most of the cockle samples were obtained. Thus, the results presented in this study do not necessarily rule out the possibility that the diarrheal cases in this region could be due to eating seafood. Nevertheless, the source of human infection still remains an important area to be explored further in Thailand.

PFGE is a reliable typing tool used for determining clonal relatedness of bacteria (Alam et al., 2009; Suffredini et al., 2011). PFGE results suggested no significant clonal links between the diverse clinical and cockle isolates. The overall PFGE and clustering results suggest that cockles obtained from markets in Nong Khai and Khon Kaen may have come mostly from Surat Thani farms 1 and 2, whereas PFGE cluster C indicates a common source for most of the diarrheal isolates and includes one sample from Surat Thani farm 2. PFGE cluster E shows that some shops in Khon Kaen markets likely purchased cockles from Surat Thani farm 3. There was no significant association between PFGE clusters and collection sites (markets and provinces), indicating that most cockles sold in the markets might have come from various farms or sources.

In this study, the majority of *V. parahaemolyticus* isolates tested (91%) were susceptible to most antimicrobial agents except for ampicillin. This is similar to results reported from Chile (Dauros et al., 2011), China (Sun et al., 2013) and Italy (Ottaviani et al., 2013). Clinical isolates exhibited more antimicrobial resistance than did the cockle isolates, possibly indicating overuse of antimicrobial agents by humans in northeastern Thailand. Although tetracycline and ciprofloxacin (Han et al., 2007), the current drugs of choice, are still effective for the treatment of *V. parahaemolyticus* infection, continuous monitoring of antimicrobial

response is necessary for efficient management of *V. parahaemolyticus* infection worldwide.

5. Conclusions

Diarrhea due to the seafood-borne pathogen *V. parahaemolyticus* has been a longstanding problem for millions of seafood lovers worldwide, including Thailand. This study provides evidence that seafood, such as cockles from coastal and estuarine waters of Thailand, might serve as a source of potentially virulent and genetically highly diverse strains of *V. parahaemolyticus* because among these are pandemic serovariants such as O3:KUT and O4:KUT. The pandemic serogroup O3:K6 was not found in Thai cockles but was common among *V. parahaemolyticus* isolated from clinical samples in Khon Kaen province, as was the pandemic serovariant O4:K68, suggesting other foods and/or seafoods other than cockles as the source of the infections. Most current antimicrobial agents in routine use are still effective.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2016.01.006.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:http://dx.doi.org/10.1016/j.meegid.2016.01.006. These data include the Google map of the most important areas described in this article.

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