MOLECULAR ANALYSIS OF *VIBRIO VULNIFICUS* ISOLATED FROM COCKLES AND PATIENTS IN THAILAND

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Abstract. *Vibrio vulnificus* can cause septicemia, wound infection and gastroenteritis. The most severe infections are related to consumption of raw or undercooked seafood. Virulence genes, biomarkers, antimicrobial resistance, and genetic relationships among *V. vulnificus* isolated from clinical and environmental sources in Thailand have not hitherto been investigated. *ViuB* encoding vulnibactin siderophore was detected in 33% and 50% of clinical and environmental (cockle) *V. vulnificus* isolates, respectively, and capsular polysaccharide allele 1 in 67% and 75% of clinical and environmental isolates, respectively. Analysis of the 16 S rDNA gene revealed that type B was the most frequent in both clinical and environmental isolates (67%) whereas the non type-able (30%) was detected only in environmental isolates. The virulence-correlated gene (*vcg*) with both type C and E together was the most frequently found among the clinical (67%) and environmental (72%) isolates. Pulsed-field gel electrophoresis differentiated *V. vulnificus* into 2 clusters; most cockle samples (83%) and all clinical isolates grouped into cluster II, indicating a possible clonal relationship between *V. vulnificus* isolated from patients and cockles. Only 20% of environmental isolates were resistant to ampicillin. These studies suggest that *V. vulnificus* isolated from cockles has virulence genes similar to those in clinical isolates and thus may have the potential of causing disease.

Keywords: *Vibrio vulnificus*, antibiotic susceptibility, virulence genes, PFGE, Thailand

INTRODUCTION

Vibrio vulnificus is a gram-negative bacterium found in marine and estuarine environments worldwide (Vickery *et al*, 2007). Consumption of raw or undercooked seafood or exposure of open wounds to contaminated water are the main causes of infection (Grau *et al*, 2008). A high mortality rate from septicemia caused by *V. vulnificus* is related to the consumption of oysters or seafood (Oliver, 2005; Jones and Oliver, 2009), especially in high risk populations, *ie*, those with liver

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disease, diabetes and/or immunocompromising diseases (Hsueh *et al*, 2004).

V. vulnificus possesses several virulence factors; *eg*, capsular polysaccharide (CPS), lipopolysaccharide, hemolysin, siderophores, exotoxins, pili and flagella (Strom and Paranjpye, 2000; Gulig *et al*, 2005). Extracellular *V. vulnificus* hemolysin A (VvhA) is encoded by *vvhA* (Wright and Morris, 1991), having hemolytic activity and cytotoxicity (Gulig *et al*, 2005). *VvhA* is used as a species-specific gene marker for detection of *V. vulnificus* (Han and Ge, 2010), but *V. vulnificus* produces vulnibactin siderophore encoded by *viuB*, which is required for iron acquisition (Litwin *et al*, 1996) and detection of *viuB* is more strongly associated with clinical than environmental isolates (Panicker *et al*, 2004).

V. vulnificus subtypes can be differentiated using sequence variations of such highly correlated biomarkers as the virulence-correlated gene (*vcg*), 16 S rDNA and CPS operon (Nilsson *et al*, 2003; Rosche *et al*, 2005; Chatzidaki-Livanis *et al*, 2006). *Vcg* has been used for the differentiation of clinical (C type) and environmental (E type) isolates (Rosche *et al*, 2005). Among the 16 S rDNA sequences, almost all environmental isolates are of the A type, whereas clinical isolates are mostly the B type (Nilsson *et al*, 2003). CPS operon can also be used to distinguish between clinical isolates (CPS allele 1) and environmental isolates (CPS allele 2) (Chatzidaki-Livanis *et al*, 2006).

Pulsed-field gel electrophoresis (PFGE) has been used to distinguish among strains from different sources (Jeong *et al*, 2011), has good discriminatory power for subtyping *V. parahaemolyticus* (Chowdhury *et al*, 2000; Martinez-Urtaza *et al*, 2004) and is used to identify genetic dissimilarities in *V. vulnificus* (Jeong *et al*, 2011).

In Thailand, cockles are mostly raised in the south and distributed around the country (Kanjanasopa *et al*, 2011; Kiratisin *et al*, 2012). A study to elucidate the relationship between clinical and environmental isolates has not yet been conducted for *V. vulnificus* in Thailand. The objectives of this study were to examine whether *V. vulnificus* isolated from clinical and environmental sources carried the same virulence-associated genes, to compare the correlation of PFGE patterns of *V. vulnificus* isolates from different sources, and to determine the antimicrobial resistance of *V. vulnificus* isolated from different sources in Thailand.

MATERIALS AND METHODS

Bacterial isolates and collection sources

A total of 44 isolates of *V. vulnificus* were examined, including 4 and 40 clinical and environmental isolates, respectively. The clinical isolates were from blood samples from Bangkok and Khon Kaen, Thailand, and *V. vulnificus* ATCC 27562 (the reference strain) was originally isolated from an American patient with a wound infection. The 40 environmental isolates were from 143 cockle samples collected from three cockle farms in Surat Thani (southern Thailand), 5 markets in Nong Khai (Northeast Thailand, bordering Lao PDR) and 5 markets in Khon Kaen (a primary hub in Northeast Thailand) (Senachai *et al*, 2013). This study was approved by institutional Human Ethics Committee (HE 551043).

Isolation and identification of *V. vulnificus*

In brief, 250 g of each cockle sample were cut into small pieces and suspended in 250 ml of phosphate-buffered saline (PBS), then 20 ml (10 g) of cockle sus-

pension was added to 80 ml of alkaline peptone water (10X APW; Oxoid, Basingtroke, Hampshire, England). The cockle suspenion in APW was incubated at 37ºC for 6 hours, and a 5 l aliquot was streaked onto thiosulfate-citrate-bilesalt-sucrose (TCBS) agar (Eiken, Tokyo, Japan) and incubated at 37ºC for 18 hours. Suspected *V. vulnificus* colonies were identified using standard biochemical tests (Ramamurthy and Nair, 2007).

PCR analysis of *V. vulnificus* virulence genes

DNA was prepared using the boiling method according to Bilung *et al* (2005). In brief, *V. vulnificus* was grown overnight in brain heart infusion (BHI) with shaking at 37ºC. Following sedimentation (4,000*g* for 5 minutes), the bacterial pellet was resuspended in sterile distilled water, boiled for 10 minutes, cooled on ice, sedimented (2,000*g* for 5 minutes) and the supernatant was used as a template for PCR assay. PCR conditions, primers and expected amplicon sizes are shown in Table 1. PCR (Veriti Thermal Cycler, Applied Biosystems, FosterCity, CA) was conducted in a total volume of 25 l containing 2.5 l of 1X PCR buffer, 200 M dNTPs, 0.3 M each primer, 0.5 U *Taq* DNA polymerase and 300 ng of genomic DNA. Amplicons were separated by 1.5% agarose-gel electrophoresis and visualized under UV light (Bio-Rad Gel™ Doc XR+ Imager) after ethidium bromide staining.

PFGE

PFGE was performed according to CDC protocol for *V. cholerae* (CDC, 2009). In brief, *V. vulnificus* DNA was digested with 40 U *Not*I (Promega, Southampton, UK) at 37ºC for 4 hours. *Salmonella* Braenderup H9812 was used as a size standard marker. PFGE was performed in 1% SeaKem Gold agarose (FMC, Lonza, Rockland, ME) in 0.5X Tris-borate-EDTA buffer using a CHEF DRIII system (Bio-Rad, Hercules, CA) at 6.0 V/cm. Pulse times were ramped in block 1 at 2-10 seconds for 13 hours and in block 2 at 20-25 seconds for 6 hours. Cluster analysis of PFGE patterns was performed using BioNumerics (Version 4.6) software. The genetic relationships were determined using Dice coefficient and dendrograms created using unweighted pair group method with arithmetic averages (UPGMA).

Antimicrobial susceptibility testing

Antimicrobial susceptibility of *V. vulnificus* was tested using disk diffusion method according to the Clinical Laboratory Standards Institute (CLSI, 2009). The commercial disks (Oxoid, Unipath, Basingstoke, England) included ampicillin (10 g), cefotaxime (30 g), chloramphenicol (30 g), ciprofloxacin (5 g), gentamicin (10 g), nalidixic acid (30 g), norfloxacin (10 g), ofloxacin (5 g), tetracycline (30 g) and trimethoprim-sulfamethoxazole (1.25/23.75 g). The control was *Escherichia coli* ATCC 25922. Diameter of inhibition zones was measured and interpreted according to CLSI requirements.

Statistical analysis

Correlations between PFGE clusters and collection sources, and PFGE clusters and virulence genes (16S rDNA and *vcg* types) were analyzed using chi-square or Fisher's exact test. A p-value <0.05 is considered statistically significant.

RESULTS

Virulence and associated genes of *V. vulnificus* isolated from patients and cockles

All clinical and environmental isolates possessed *vvh*A, confirming that all isolates were *V. vulnificus*. The *viuB*, CPS

Table 1
Primers and amplicons used in PCR detection of species-specific and virulence genes of *V. vulnificus*.

Gene	Amplicon size (bp)	Primer sequence	PCR condition	Reference
*vvh*A	205	F-5′- TTCCAACTTCAAACCGAACTATGAC -3′ R-5′- ATTCCAGTCGATGCGAATACGTTG -3′	94°C, 30 sec; 60°C, 30 sec; 72°C, 45 sec (35 cycles)[a]	Brasher *et al*, 1998
*viu*B	504	F-5′- GGTTGGGCACTAAAGGCAGATATA -3′ R-5′-CGGCAGTGGACTAATACGCAGC -3′		Panicker *et al*, 2004
CPS allele1	342	F-5′-TTTGGGATTTGAAAGGCTTG -3′ R-5′-GTGCCTTTGCGAATTTTGAT -3′	94°C, 1 min; 50°C, 1 min; 72°C, 1 min (35 cycles)[b]	Han *et al*, 2009
CPS allele 2	152	F-5′-′TTCCATCAAACATCGCAGAA -3′ R-5′-′CTTTTGTCCGGCTTCTATCG -3′		Han *et al*, 2009
16S rDNA type A	893	F-5′- AGCTTCGGCTCAAAGAGG -3′ R-5′-CCAGCGTCTCCGCTAGAT -3′		Han and Ge, 2010
16S rDNA type B	893	F-5′-GCCTACGGCCCAAAGAGG -3′ R-5′-CCTGCGTCTCCGCTGGCT -3′		Warner *et al*, 2008a
*vcg*C	99	F-5′- AGCTGCCGATAGCGATCT -3′ R-5′-TGAGCTAACGCGAGTAGTGAG -3′		Modified from Warner *et al*, 2008b
*vcg*E	278	F-5′-CTCAATTGACAATGATCT -3′ R-5′-CGCTTAGGATGATCGGTG -3′		Modified from Rosche *et al*, 2005

[a]PCR condition for *vvh*A and *viu*B; [b]PCR condition for CPS allele 1, CPS allele 2, *vcgC*, *vcgE*, 16S rRNA type A and type B.

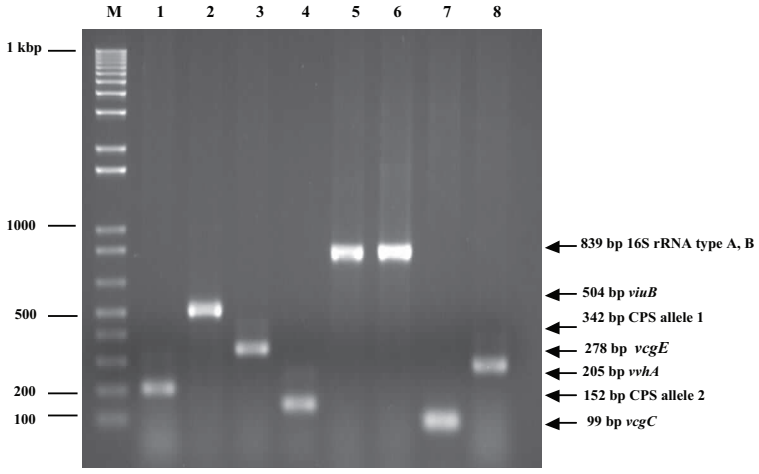


Fig 1–PCR amplicons of the virulence genes in *V. vulnificus*. Lane M, 1 kb size marker; lane 1, *vvh*A, a species-specific gene (205 bp); lane 2, *viuB* gene (504 bp); lane 3, CPS allele1 (342 bp); lane 4, CPS allele 2 (152 bp); lane 5, *vcgC* (99 bp); lane 6, *vcgE* (278 bp); lane 7, 16 S rDNA type A (839 bp); lane 8, 16 S rDNA type B (839 bp).

allele 1 and CPS allele 2 were detected by PCR in 33, 67 and 33% of the clinical isolates compared to 50, 75 and 22%, respectively in the environmental isolates (Fig 1). The 16 S rDNA analysis revealed that 67% of clinical isolates were type B while 33% were type AB, whereas 67% of environmental isolates were type B, 2% type AB and 30% un-type-able. The most prevalent type of *vcg* was type C and E, detected in 67% and 72% of the clinical and environmental isolates, respectively (Table 2).

PFGE of *V. vulnificus* isolated from patients and cockles

Twenty-two *V. vulnificus* isolates, including 4 clinical (from 3 Thai patients and 1 reference strain) and 18 environmental (from cockles from Surat Thani, Nong Khai and Khon Kaen) isolates, were selected for differentiation using PFGE, which comprised 12-21 bands with individual banding patterns (Fig 2). *V. vulnificus* isolates could be subdivided into 2 clusters (I and II) at 50% similarity level based on a cluster analysis (Fig 2). The majority of *V. vulnificus* isolates (86%) came from patients and cockles (cluster II) while 3 isolates (14%) came from cockles purchased at Khon Kaen market (cluster I). A significant association was revealed between PFGE clusters and collection sites (markets and provinces) ($p = 0.023$), but there is no association between *V. vulnificus* clusters and virulence factors (*viuB* and CPS).

Antimicrobial susceptibility testing

All *V. vulnificus* isolated from patients and the majority (80 %) of cockle isolates were sensitive to all antimicrobial agents, except 8 of the latter that were resistant to ampicillin.

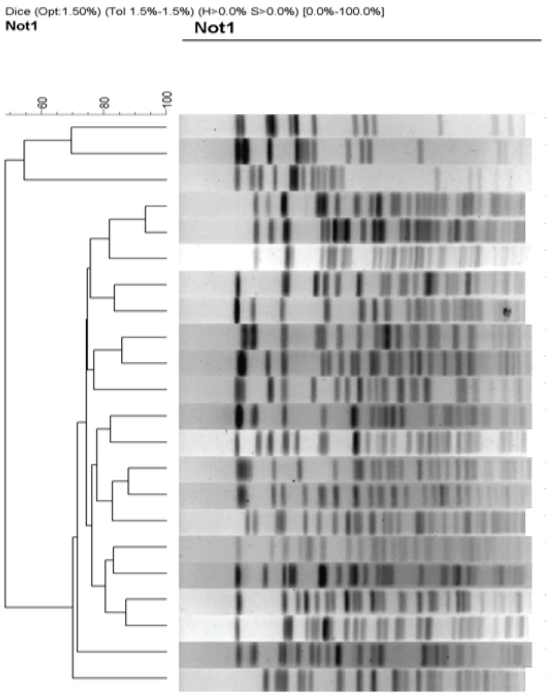
DISCUSSION

We investigated 44 isolates of *V. vulnificus* in Thailand to understand its pathogenesis, antimicrobial resistance and genetic relationship between clinical and environmental sources (cockles collected from 3 cockle farms in Surat Thani, and samples from markets in Nong Khai and Khon Kaen).

One-third of the *V. vulnificus* clinical isolates and 50% of the environmental ones were positive for *viuB*, and detection of CPS allele 1 was slightly higher (75%) among environmental isolates than the clinical (67%) isolates. Although the number of clinical specimens was small, our results did not correspond to previous studies. In Japan *viu*B is more

Table 2
Distribution of virulence genes in *V. vulnificus* isolated from patients and environment.

Source	Number of isolates	Species-specific gene (%)	Virulence gene (%)	Biomarkers (%)									
				CPS		16S rRNA type				*vcg* type			
		vvhA	*viuB*	CPS1	CPS2	A	B	AB	Non-typeable	C	E	CE	Non-typeable
ATCC 27562 ($n = 1$)	1	+	+	-	+	+	-	-	-	-	+	-	-
Clinical isolates	1	+	-	-	+	-	+	-	-	-	-	+	-
($n = 3$)	1	+	+	-	+	-	+	-	-	-	-	+	-
	1	+	+	+	-	-	-	+	-	+	-	-	-
Total	3	3 (100)	1 (33)	2 (67)	1 (33)	0	2 (67)	1 (33)	0	1 (33)	0	2 (67)	0
Environmental	9	+	+	+	-	-	+	-	-	-	-	+	-
isolates ($n = 40$)	6	+	-	+	-	-	+	-	-	-	-	+	-
	5	+	-	+	+	-	+	-	-	-	-	+	-
	2	+	+	-	-	-	+	-	-	-	-	+	-
	2	+	-	+	-	-	-	-	+	-	-	-	+
	2	+	+	+	-	-	-	-	+	-	-	-	+
	2	+	+	-	-	-	-	-	+	-	-	-	+
	2	+	-	-	-	-	-	-	+	-	-	+	-
	2	+	-	-	-	-	-	-	+	-	-	-	+
	1	+	+	+	+	-	+	-	-	-	-	-	+
	1	+	+	+	+	-	+	-	-	+	-	-	+
	1	+	+	+	-	-	+	-	-	+	-	-	-
	1	+	+	+	-	-	-	-	+	-	-	+	-
	1	+	-	+	+	-	-	-	+	-	-	+	-
	1	+	+	+	-	-	-	+	-	-	-	+	-
	1	+	-	-	+	-	+	-	-	-	-	+	-
	1	+	-	-	-	-	+	-	-	-	-	+	-
Total	40	40 (100)	20 (50)	30 (75)	9 (22)	0	27 (67)	1 (2)	12 (30)	2 (5)	0	29 (72)	9 (22)



Strain	Source	Place	
Vv 10	Cockle	Khon Kaen 1	I
Vv 12	Cockle	Khon Kaen 5	
Vv 11	Cockle	Khon Kaen 1	
Vv 6	Cockle	Surat Thani 2	II
Vv 8	Cockle	Khon Kaen 3	
Vv 1	Wound	ATCC 27562	
Vv 4	Blood	Khon Kaen	
Vv 9	Cockle	Nong Khai 5	
Vv 14	Cockle	Nong Khai 3	
Vv 16	Cockle	Surat Thani 3	
Vv 13	Cockle	Nong Khai 1	
Vv 20	Cockle	Khon Kaen 2	
Vv 3	Blood	Bangkok	
Vv 18	Cockle	Surat Thani 3	
Vv 19	Cockle	Nong Khai 2	
Vv 5	Cockle	Surat Thani 1	
Vv 15	Cockle	Nong Khai 5	
Vv 22	Cockle	Khon Kaen 4	
Vv 17	Cockle	Surat Thani 2	
Vv 2	Blood	Bangkok	
Vv 21	Cockle	Khon Kaen 2	
Vv 7	Cockle	Nong Khai 4	

Fig 2–PFGE-*Not*I restriction patterns for *V. vulnificus* isolated from patients and cockles. Dendrogram designated by Bionumeric software illustrates the relationships between the isolates. The number following each place represents the market in that province.

commonly detected among clinical than environmental isolates (Yokochi *et al*, 2013). In our study, most of the environmental isolates possessed *viu*B and the CPS operon, supporting the hypothesis that human-pathogenic *V. vulnificus* and environmental *V. vulnificus* strains cannot be distinguished because some of the latter strains can become fully virulent in susceptible humans (Strom and Paranjpye, 2000).

The 16 S rDNA type is correlated with virulence and source (Nilsson *et al*, 2003). Our results revealed that 16 S rDNA type B predominated in both clinical (67%) and environmental (67%) isolates and nontypeable types were found in only environmental isolates. Type A 16 S rDNA was only found in *V. vulnificus* ATCC 27562. Likewise, Kim and Jeong (2001) reported that 65% of environmental isolates were of 16 S rDNA. In our study, *vcg* type C and E together were the most common in both clinical and environmental isolates, while the E type alone was not found in environmental isolates. Our results are in disagreement with a previous study that reported 90% of clinical isolates are *vcg* type C, while 93% of environmental isolates *vcg* type E (Bogard and Oliver, 2007), indicating that *V. vulnificus* may transfer genes among clinical and environmental strains. There is no significant difference among genotypes isolated from clinical and environmental sources, corresponding to a previous study that suggested the discrepancies in genotypes may be due to differences in geographic regions, even

though from the same country (Yokochi *et al*, 2013).

Although our results showed that both clinical and environmental isolates possessed many virulence genes however, diseases caused by *V. vulnificus* may depend on other factors, such as dose of infection and host factors, rather than simply on the specific types of bacterial isolates (Jones and Oliver, 2009). Additionally, there are no unique virulent biomarkers to differentiate among clinical and environmental isolates because those isolates showed equal virulence in animals and cell culture models (DePaola *et al*, 2003).

PFGE distinguished the 4 clinical and 18 environmental isolates of *V. vulnificus* into 2 clusters. All isolates showed individual PFGE profiles with only 50% PFGE similarity, indicating that there is a high genetic diversity within the subgroups of *V. vulnificus* (Tamplin *et al*, 1996). PFGE cluster I was found in 3 cockle isolates purchased from 2 Khon Kaen markets (Khon Kaen markets 1 and 5) whereas PFGE cluster II was associated with the remaining isolates. The significant association between PFGE clusters and collection sites (markets and provinces) indicated that the 3 isolates of PFGE in cluster I might have come from the same farm at the same time. Similarly, as most of the cockle isolates (15/18 samples; 83%) and all the clinical isolates (3 Thai isolates and 1 American strain) were in cluster II, this indicates that they may have originated from a related clone.

In Thailand, several bacteria including the genus *Vibrio* have become resistant to antimicrobial agents (Chomvarin *et al*, 2013). *V. vulnificus*, as shown in this and previous studies (Han *et al*, 2007), is still susceptible *in vitro* to many antimicrobial agents. Thus, the current antimicrobial drugs used are effective for the treatment of *V. vulnificus* infection. As the most common diseases caused by *V. vulnificus* are septicemia and wound infection (Jones and Oliver, 2009), prompt antimicrobial treatment is recommended (Bross *et al*, 2007; Han *et al*, 2007). Ceftazidime in combination with doxycycline and ciprofloxacin with cefotaxime has also been effective (Bross *et al*, 2007; Horseman and Surani, 2011).

In summary, *V. vulnificus* isolated from cockles in Thailand possess many virulence genes that have disease-causing potential. There is a genetic relationship between *V. vulnificus* isolated from patients and cockles. The current antimicrobial agents in routine use are reassuringly still effective.

ACKNOWLEDGEMENTS

This study was supported by a grant from the National Research University Project (NRU), Khon Kaen University, Thailand. The authors thank Professor Yukifumi Nawa for suggestions in the preparation of the manuscript, staff of the Clinical Microbiology Laboratory Unit, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University and Khon Kaen Hospital for collecting the clinical bacterial isolates and Mr Bryan Roderick Hamman for assistance with the English of the manuscript.

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