DISTRIBUTION AND SEQUENCE ANALYSIS OF VIRULENCE ASSOCIATED GENES IN *VIBRIO CHOLERAE* O1, O139 AND NON-O1/NON-O139 ISOLATES FROM THAILAND

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Abstract. Virulence-associated genes of *Vibrio cholerae* including O1, O139 and non-O1/non-O139 from an outbreak in Songkhla Province and sporadic cases occurred in Thailand during 1993 - 2002 were investigated. One hundred eighty-five *V. cholerae* strains were examined for the presence of virulence-associated genes including *ctxA*, *tcpA*, *zot*, *toxR*, *toxS*, *toxT*, and *ace* by polymerase chain reaction. DNA sequences of *ctxA*, *tcpA*, *zot* and *toxR* also were investigated in 8 selected isolates. Results showed that the virulence factors genes were distributed in the majority of *V. cholerae* O1 biotype Inaba and Ogawa strains (85-97%). All 6 strains of the O139 harbored *toxR*, *toxS* and *toxT* whereas *ctxA*, *tcpA*, *zot* and *ace* were detected only 50-67%. Toxins genes found in non-O1/non-O139 strains ranged 8-30% except *toxR* (73.5%). Results of multiple sequence alignments among the isolates compared with *V. cholerae* O1 in database (embl M21249), showed that *ctxA*, *tcpA* and *zot* sequences in all 8 isolates were conserved, but base changes were found in *toxR* sequence. These molecular characteristics of *V. cholerae* isolated from Thailand will provide detailed information for facilitating future studies on the development and design of appropriate vaccine providing protection against local strains.

INTRODUCTION

Diarrheal disease caused by *Vibrio cholerae* is a clinical-epidemiologic syndrome. The etiologic agent usually belongs to serogroup O1 biotype El Tor and serogroup O139 (Albert *et al*, 1997). This disease occurs in many developing countries and has resulted in a large number of deaths. A rapid spread of epidemic cholera due to a new serogroup (O139) giving the same symptoms as characterized in *V. cholerae* serogroup O1 has been suggested(CDC, 1993; Cholera Working Group, 1993; Ramamurthy *et al*, 1993). *V. cholerae* non-O1/non-O139 serogroups have increasingly been recognized as the causative agents of sporadic cases of cholera-like disease (Morris, 1994) and outbreaks (Bagchi *et al*, 1993; Morris, 1994; Dalsgaard *et al*, 1995). *V. cholerae* non-O1/non-O139 was suggested to be involved in the emergence of a new variant of *V. cholerae* by horizontal gene transfer (Bik *et al*, 1995).

V. cholerae harbors a virulence regulon

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consisting of genes involved in colonization, toxin production and bacterial survival within the host (Miller and Mekalanos, 1988; Peterson and Mekalanos, 1988; Xu *et al*, 2003). The presence of *ctx* encoding cholera toxin (CT) clearly shows that it plays an important role in pathogenicity. However, diarrheal disease with mild to moderate symptoms was still observed in many individuals who had deletion of genes encoding CT of *V. cholerae* strains (Levine *et al*, 1988; Tacket *et al*, 1997). In addition, a cholera toxin gene in non-O1/non-O139 strains was suggested to be potential to cause cholera-like disease (Dalsgaard *et al*, 1998). Other toxins include Zot (Fasano *et al*, 1991), Ace (Trucksis *et al*, 1993) and hemolysin/cytolysin (Yamamoto *et al*, 1986). Ace and Zot appear to be associated with *ctx* gene and may be responsible for potential pathogenic roles of cholera infection (Trucksis *et al*, 1993). The hemolysin/cytolysin was found in both pathogenic and non-pathogenic strains of *V. cholerae* and has no significant association with *ctx* (Kaper *et al*, 1995). The pili colonization factor, TCP, in *V. cholerae* O1 is involved in human pathogenicity (Taylor *et al*, 1987). Members of the regulatory cascade, including ToxR, ToxS and ToxT are involved in activating transcription required for co-ordinate expression of genes associated with pathogenicity in *V. cholerae* (Champion *et al*, 1997).

During 1992-1996, sporadic cholera cases in 14 provinces of southern Thailand were reported by the Division of Epidemiology, Ministry of Public Health, Thailand. Later in 1998, an unusually high incidence of cholera cases in southern Thailand was noticed. The strains were characterized for molecular epidemiologic analysis (Kondo *et al*, 2001)indicating that cholera cases in Thailand in the 1990s were caused by *V. cholerae* O1 El Tor biotype Ogawa serotype El Tor Ogawa. Some *V. cholerae* O139 were isolated between 1993 and 1995 (Dalsgaard *et al*, 1998; Hoge *et al*, 1996). In 2001, another outbreak due to *V. cholerae* O1 El Tor biotype Inaba serotype took place in Songkhla Province of southern Thailand. During this period of time other sporadic cases were also isolated in other regions of Thailand. These isolates have not been fully explored.

In this study, we characterized at the molecular level the strains of *V. cholerae* O1, O139 and non-O1/non-O139 from outbreaks and sporadic cases isolated in Thailand during 1996 to 2002. The distribution of virulence-associated genes of the *V. cholerae* strains and the genes sequences were investigated in order to gain detailed information for a better understanding of the presence of virulence-associated factors in the *V. cholerae* strains isolated in Thailand.

MATERIALS AND METHODS

Samples

One hundred eighty-five *V. cholerae* strains were collected from different regions of Thailand (Table 1). *V. cholerae* strains were confirmed by standard biochemical tests and serotyping using polyvalent anti-O1 and O139 antiserum and antiserum specific to serogroup Ogawa and Inaba (Kelly *et al*, 1991). All isolates were examined for presence of virulence-associated genes, including *ctxA, tcpA, zot, toxR, toxS, toxT* and *ace*. *V. cholerae* isolates were selected for *ctxA, tcpA, zot* and *toxR* sequence analysis. Five strains were *V. cholerae* El Tor Inaba O1 isolated in 2001 from an outbreak in Songkhla Province (n = 4) and from Phuket Province (n = 1). Three strains were *V. cholerae* O1 El Tor Ogawa (isolated in 1999), O139 (2001) and non-O1/non-O139 (2001).

Detection of virulence-associated genes by polymerase chain reaction (PCR)

The presence of genes encoding *ctxA, tcpA, zot, toxR, toxS, toxT* and *ace* in test strains were examined by PCR as previously

Table 1
V. cholerae O1, O139 and non-O1/non-O139 serogroups from different regions of Thailand.

Serotype (no.)	Region (no.)				Period of isolation
	North (7)	South (150)	North-East (11)	Central (17)	
O1 El Tor Inaba (133)	6	108	9	10	2000-2002
O1 El Tor Ogawa (11)	1	8	1	1	1999-2002
O139 (6)	-	1	1	4	1993-2002
Non-O1/Non-O139 (35)	-	33	-	2	1999-2002

Table 2
Primer pairs for virulence-associated genes detection used in the study.

Target gene	Nucleotide sequence (5'-3')	Amplicon size (bp)	Reference
ctxA	CTC AGA CGG GAT TTG TTA GGC ACG TCT ATC TCT GTA GCC CCT ATT ACG	564	Keasler and Hall, 1993
tcpA	GAA GAA GTT TGT AAA AGA AGA ACA C GAA AGG ACC TTC TTT CAC GTT G	618	Keasler and Hall, 1993
zot	TGG CTT CGT CTG CTG CCG GCG ATT CAC TTC TAC CCA CAG CGC TTG CGC	1,083	Aidara *et al*, 1998
toxR	CGG GAT CCA TGT TCG GAT TAG GAC AC CGG GAT CCT ACT CAC ACA CTT TGA TGG C	900	Ghosh *et al*, 1997
toxS	CCA CTG GCG GAC AAA ATA ACC AAC AGT ACC GTA GAA CCG TGA	640	Shinoda *et al*, 2004
toxT	TTG CTT GGT TAG TTA TGA GAT TTG CAA ACC CAG ACT GAT AT	581	Shinoda *et al*, 2004
ace	TAA GGA TGT GCT TAT GAT GGA CAC CC CGT GAT GAA TAA AGA TAC TCA TAG	314	Aidara *et al*, 1998

described with some modifications (Shirai *et al*, 1991). The specific oligonucleotide primer pairs and expected sizes of amplicons are listed in Table 2. PCR was carried out in a DNA thermal cycler with conditions as follows: initial denaturation at 94°C for 4 minutes, followed by 30 cycles of 94°C for 1 minute and 60°C for 1.5 minutes and 72°C for 1.5 minutes and a final extension at 72°C for 10 minutes. Amplicons were separated by 1% agarose gel-electrophoresis and visualized by ethidium bromide staining and UV transillumination. Negative PCR results were repeated for confirmation.

Nucleotide sequencing

CtxA, *tcpA*, *zot* and *toxR* genes from 8 strains including *V. cholerae* O1 Inaba and Ogawa serotypes, O139 and non-O1/non/O139 were sequenced commercially using 3100 Genetic analyzer (ABI). The sequences were analyzed with BLAST and multiple alignment of sequences was generated with

Table 3
Genotype of *V. cholerae* O1 and O139 serogroup found in the study.

Genotype	*ctxA*	*tcpA*	*zot*	*toxR*	*toxS*	*toxT*	*ace*	No. of strains (%)		
								O1 Inaba	O1 Ogawa	O139
1	+	+	+	+	+	+	+	48 (39)	5 (45.5)	1 (16.7)
2	+	+	+	-	+	+	+	13 (10)	1 (9.1)	0 (0)
3	+	+	+	+	+	+	-	13 (10)	1 (9.1)	1 (16.7)
4	+	+	+	+	+	-	+	9 (7.3)	0 (0)	1 (16.7)
5	+	+	+	+	-	-	+	8 (6.5)	0 (0)	0 (0)
6	+	+	+	+	-	+	+	6 (4.9)	3 (27.3)	0 (0)
7	+	+	+	+	-	-	-	4 (3.3)	0 (0)	0 (0)
8	+	+	+	+	+	-	-	4 (3.3)	0 (0)	0 (0)
9	+	+	-	+	+	+	+	3 (2.4)	0 (0)	0 (0)
10	+	-	+	+	+	+	+	2 (1.6)	0 (0)	0 (0)
11	+	+	+	-	+	-	+	2 (1.6)	0 (0)	0 (0)
12	+	+	+	+	-	+	-	1 (0.8)	0 (0)	0 (0)
13	+	+	+	-	+	+	-	1 (0.8)	0 (0)	0 (0)
14	+	+	+	-	+	-	-	1 (0.8)	0 (0)	0 (0)
15	-	+	+	+	+	+	+	1 (0.8)	0 (0)	0 (0)
16	+	+	-	+	+	-	+	1 (0.8)	0 (0)	0 (0)
17	+	-	-	+	+	-	+	1 (0.8)	0 (0)	0 (0)
18	+	-	-	+	-	+	-	1 (0.8)	0 (0)	0 (0)
19	-	-	-	+	+	+	+	1 (0.8)	0 (0)	0 (0)
20	-	-	-	+	+	-	-	1 (0.8)	1 (9.1)	1 (16.7)
21	-	+	-	+	+	+	+	0 (0)	0 (0)	1 (16.7)
22	-	-	-	+	+	+	-	0 (0)	0 (0)	1 (16.7)

program Clustal W (Thompson *et al*, 1994) and optimized manually.

RESULTS

Distribution of virulence-associated genes in *V. cholerae*

The majority of the *V. cholerae* O1 strains including Inaba and Ogawa carried *ctxA*, *zot* and *tcpA*. These three genes were detected also in O139 (50-67%) and non-O1/non-O139 strains (9-15 %). Other toxins genes including *toxR*, *toxS* and *toxT* were found mainly in the O1 and O139 strains. On the other hand, most of the non-O1/non-O139 strains contained *toxR* (74%), while *toxS*, *toxT* and *ace* were detected only 8-30% of the strains (data not shown).

All *V. cholerae* O1 El Tor Inaba strains isolated from the outbreak in Songkhla during 2001 were positive for both *ctxA* and *zot*. *V. cholerae* O1 El Tor Ogawa strains from the outbreak in Phuket and sporadic cases from other regions were *ctxA* and *zot* positive except one strain that was *ctxA* and *zot* negative. Three *V. cholerae* O139 strains harbored *ctxA* and *zot* gene. Six strains of *V. cholerae* O1 El Tor Inaba strains from sporadic cases and 2 out of 35 non-O1/non-O139 strains were positive for *ctxA* but negative for *zot*.

Twenty-two genotypes of *V. cholerae* O1 and O139 serogroup are listed in Table 3.

Genotype 1 was observed most frequently in *V. cholerae* O1 Inaba (39%) and Ogawa (45.5%). Genotypes 1, 3 and 20 were found in both O1 and O139 serogroups. Interestingly some *V. cholerae* non-O1/non-O139 strains also belonged to genotype 20 (4 of 35) and genotype 12 (1 of 35), which were detected in O1 and O139 serogroups. The genotype found in most *V. cholerae* non-O1/non-O139 strains was *toxR*$^+$ (data not shown).

Virulence-associated gene sequence analysis

Analysis by multiple alignment of nucleotide sequences revealed that *ctx, tcpA* and *zot* sequences were identical among the test strains with *Vibrio cholerae* strains in database (data not shown), confirming that the

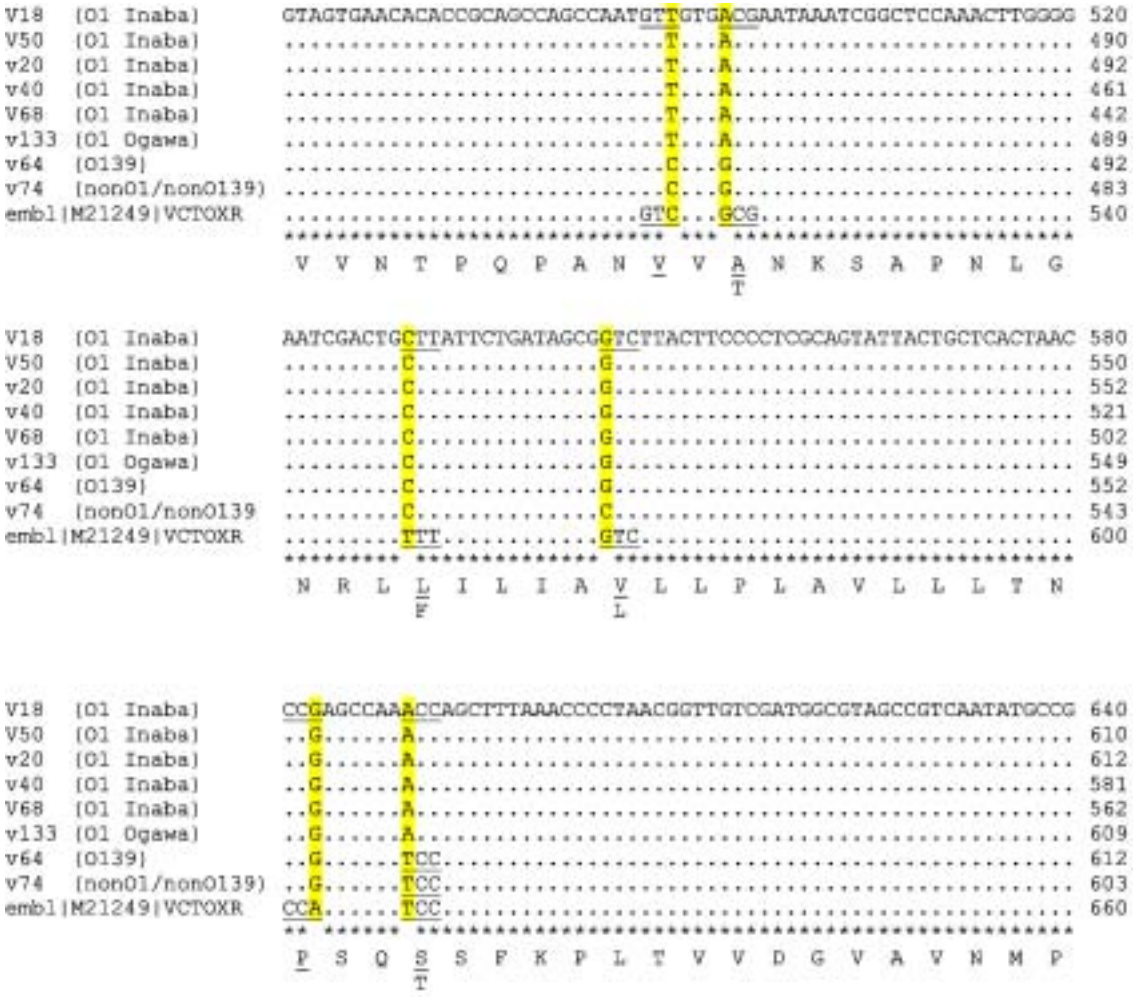

Fig 1–Multiple sequence alignment of *toxR* from selected *V. cholerae* strains. The nucleotide sequence is numbered on the right. The predicted amino acid sequences of embl | M21249 | VCTOXR are indicated below the nucleotide sequences. The different nucleotides are shaded and different amino acid sequences are underlined. Identical sequences are indicated by asterisks.

regions of these three genes examined in this study were conserved. However, nucleotide sequences of *toxR* had base changes among the isolates compared with *V. cholerae* strain in database (embl M21249). Base changes that showed different predicted amino acids were observed between nucleotide position 517 and 615 (Fig 1). At nucleotide position 517-519, the amino acid of *V. cholerae* O1 El Tor Inaba serotype was threonine whereas it was alanine in *V. cholerae* O1 El Tor Ogawa serotype, O139, non-O1/non-O139 and embl M21249 strain. Phenylalanine at nucleotide position 553-555 was present in all test strains but was leucine in the database strain. Most of the test strains and database sequence strain at position 568-570 was valine while only non-O1/non-O139 it was leucine. Position 613-615 in O1 Inaba and Ogawa strains encoded serine but threonine in O139 and non-O1/non-O139.

DISCUSSION

The pathogenicity of *V. cholerae* O1 and O139 strains depends on CT encoded by *ctxA* and colonization factor encoded by *tcpA* for the ability of adhesion and colonization of small intestine (Herrington *et al*, 1988). *V. cholerae* O1 and O139 that produce CT are causative agents of epidemic cholera. The O1 strains that do not produce toxin and non-O1/non-O139 are also associated with clinical symptoms such as cholera, gastroenteritis, septicemia and extraintestinal infections (Morris, 1994; Rodrigue *et al*, 1994; Saha *et al*, 1996; Rivera *et al*, 2001). Interestingly, we found that CT and TCP (*tcpA*) were present in non-O1/non-O139 strains isolated in 2001, whereas the absence of CT in the non-O1/non-O139 *V. cholerae* strains isolated during 1980 -1982 in Thailand was reported (Hanchalay *et al*, 1985). The transfer of virulence-associated genes from toxigenic *V. cholerae* strains may occur among non-O1/non-O139 strains as suggested in a previous report (Waldor and Mekalanos, 1996).

The *V. cholerae* O1 El Tor Inaba strains isolated from an outbreak in Songkhla Province in 2001 showed interesting genotypes among the strains. We found that the *V. cholerae* O1 El Tor Inaba and non-O1/non-O139 *V. cholera* strains harbored *ctxA* but not *zot*. This result has been reported previously (Rivera *et al*, 2001). However, other reports showed the presence of *zot* and absence of *ctx* in *V. cholera* and *V. minicus* (Karasawa *et al*, 1993; Chowdhury *et al*, 1994; Ghosh *et al*, 1997). The *zot* gene encoding ZOT is involved in increasing permeability of the small intestinal mucosa (Fasano *et al*, 1991), and it was suggested that *zot* represents a new mechanism if CT is deleted but still causes diarrhea. In this study, the non-O1/non-O139 strains which have *zot* alone would therefore be potentially pathogenic.

In addition, toxin co-regulated pilus (TCP) and the regulation of virulence genes including *toxR*, *toxS* and *toxT* coordinate CT in the pathogenicity of *V. cholerae* (Taylor *et al*, 1987; Said *et al*, 1995; Ghosh *et al*, 1997). In this study, *tcpA* was found in most of the toxigenic *V. cholerae* O1 El Tor Inaba and Ogawa and *V. cholerae* O139 strains. However, *tcpA* gene was also found in non-O1/non-O139 strains. These results were in agreement with previous reports (Chakraborty *et al*, 2000; Nandi *et al*, 2000; Rivera *et al* 2001). It was suggested that the strains probably are able to colonize the human intestine and become toxigenic as the result of their selective advantage over nonpathogenic strains (Karaolis *et al*, 1999).

It was noticed that t*oxR* was most frequently observed in all *V. cholerae* including O1, O139 and non-O1/non-O139. *ToxR*, *toxS* and *toxT* were suggested to be involved in regulation of virulence of *V. cholerae*. DiRita

et al (1991) suggested that only *toxR* is not enough for expression of virulence genes other than *ctxAB*. *ToxT* assists in activating many *toxR*-regulated genes including *tcpA* and *ctxAB*. This study showed that the presence of these three toxin genes, but not *ctx* and *tcpA*, was observed in genotype 19, indicating that *toxT* is likely to play an important role for other virulence-associated genes of *V. cholerae*.

V. cholerae virulence cassette genes include *ctx*, *zot* and *ace*. *Ace*, located immediately upstream of *zot* and *ctx* genes, was shown to play a potential pathogenic role (Trucksis *et al*, 1993). Genotypes 20 and 22 showed the absence of the virulence cassette genes and the presence of *toxR* and *toxS*. However, as the strains were isolated from patients who had diarrhea, the symptom probably was caused by other toxin genes which are potentially regulated by *toxR* and *toxS*. Our results showed non-O1/non-O139 strains harbored *ctx*/*zot* (n = 1), *ctx* (n = 1), *zot* (n = 1) and *ace* (n = 2) indicating their potential role in the pathogenicity of diarrheal disease, while other non-O1/non-O139 are likely to be pathogenic by other virulence factors other than the genes tested in this study.

The distribution of the virulence-associated genes showed that O1 and O139 strains were mostly toxigenic. As there was no productivity of toxins in most of the non-O1/non-O139 strains, they are unlikely to have epidemic potential. However, some non-O1/non-O139 strains contained cholera toxin genes. Identical *ctxA*, *tcpA* and *zot* gene sequences of *V. cholerae* O1 and non-O1/non-O139 strains and the presence of genotypes 12 and 20 in *V. cholerae* O1 and non-O1/non-139 (data not shown) are probably due to a conversion mechanism of non-O1/non-O139 to O1 as shown in previous studies (Colwell *et al*, 1995).

None of the tested virulence-associated genes was found in three isolates of *V. cholerae* non-O1/non-O139. These toxigenic strains, which caused diarrhea in humans may be producing small quantities of the toxin products (Craig *et al*, 1981). However, further investigations for other pathogenic bacteria that can cause diarrhea is recommended to rule out other etiologic agents.

Changes in amino acids of *toxR* may lead to the changes of the expression of toxin genes, which could subsequently affect the severity of cholera infection. However, further investigations of their effects on the virulence gene expression should be conducted.

In summary, the results of the distribution of virulence-associated genes and their sequences have provided detailed information regarding virulence-associated genes among different serogroups of local Thai strains. The possible emergence of a new virulent variant strain must be carefully observed in order to be able to provide an effective control of cholera infections and outbreaks. Moreover, the sequence data will provide insightful knowledge for facilitating further studies to develop a safe vaccine against cholera infection.

ACKNOWLEDGEMENTS

This work was financially supported by Thailand Tropical Diseases Research Programme (T-2), BIOTEC/NSTDA, TRF, and TDR/WHO. We thank Dr Prasit Palittapongarnpim for his kind advice and giving us the opportunity to undertaken research study on *Vibrio cholerae*. The facilities provided by Division of Microbiology, Faculty of Medicine, Prince of Songkla University and Faculty of Medicine, Thammasat University, Thailand are gratefully appreciated.

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