MOLECULAR ANALYSIS AND ANTIMICROBIAL RESISTANCE OF *VIBRIO CHOLERAE* O1 IN NORTHEASTERN THAILAND

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Abstract. A total of 84 clinical *Vibrio cholerae* O1 isolates were collected from Khon Kaen (KK), Udon Thani (UT), Loei (LI), and Nong Khai (NK), northeastern Thailand during cholera outbreaks in 2007 and 2008. The majority of *V. cholerae* O1 strains carried nearly all the virulence-associated genes (*ctxA, zot*, and *ace*) except for four isolates and one isolate from UT and NK, respectively, which carried only *tcpA, ompU, hlyA* and *toxR.* None of the *V. cholerae* O1 strains carried *sto*. Pulsed field gel-electrophoresis (PFGE) profiling of 16 randomly chosen isolates showed the same PFGE pattern, except for one NK isolate, which was sensitive to all seven antibiotics used in the antimicrobial susceptibility tests. The tests revealed that multi-drug resistance to tetracycline and co-trimoxazole were present in KK strains (92%), followed by LI (75%) and UT (52%) strains. All strains were sensitive to norfloxacin but intermediate resistance to ciprofloxacin was found in a single strain from KK and LI. Differences in antimicrobial resistance among *V. cholerae* strains with the same PFGE pattern reflect differences in the antimicrobial agents used in each area of northeastern Thailand.

Keywords: *Vibrio cholerae* O1, antimicrobial resistance, virulence associated gene, PFGE pattern, Thailand

INTRODUCTION

Vibrio cholerae is the causative agent of cholera and is frequently found in many developing countries including Thailand (Radu *et al*, 2002; Qu *et al*, 2003). Serogroups O1 and O139 have the highest association with cholera (Sharma *et al*, 1998).

The mechanism of pathogenicity of *V. cholerae* O1 and O139 depends on the presence of virulence-associated genes located at (at least) three major pathogenicity "islands". They include the CTX genetic element, such as *ctxA, B* encoding

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cholera toxin, *zot* encoding zonula occluden toxin and *ace* encoding accessory cholera toxin (Fasano *et al*, 1991; Baudry *et al*, 1992; Trucksis *et al*, 1993). *V. cholerae* pathogenicity island (VPI), encoding toxin co-regulated pilus (TCP), type IV pilus that plays a role in colonization and as CTXϕ receptor (Karaolis *et al*, 1998), and RTX toxin gene cluster encoding a presumptive cytotoxin, an acyl transferase that is associated with ATP-binding cassette transporter system (DiRita, 2000). Other virulence genes reported are *hlyA* encoding El Tor hemolysin (Yamamoto *et al*, 1984), *stn/sto* encoding a heat stable enterotoxin (Sperandio *et al*, 1996), *ompU* encoding an outer membrane protein (Sperandio *et al*, 1996), and *toxR* encoding ToxR regulatory protein (Miller *et al*, 1987).

In the 2000s, cholera cases in Thailand were due to the *V. cholerae* O1 El Tor biotype and both the Ogawa and Inaba serotypes. Before 2004, *V. cholerae* O1 isolated from patients in 28 hospitals in central Thailand was susceptible to tetracycline and norfloxacin, the most frequently used antimicrobial agents for the treatment of cholera (Supawat *et al*, 2009). However, since then tetracycline resistance has been reported in an epidemic in southern Thailand (Kondo *et al*, 2001).

Cholera outbreaks have occurred sporadically for many years in northeastern Thailand (Bureau of Epidemiology, 2007), but it was unclear whether the outbreaks were due to a single clone, had derived from the same or different strains and the problem of multidrug resistance has not been reported before 2007 (Bureau of Epidemiology, 2007).

Laboratory diagnosis and epidemiological surveillance of cholera are based on phenotypic characteristics, including biochemical tests, antigenic properties, and antibiogram, which are known to be of limited value for predicting the epidemiological potential of *V. cholerae* strains (Stroecher *et al*, 1992; Leal *et al*, 2004). Molecular typing techniques, such as random amplified polymorphic DNA (RAPD), pulsed field gel electrophoresis (PFGE), ribotyping and multilocous enzyme electrophoresis (MEE), have been employed to study genetic relatedness (Bhowmick *et al*, 2007), but PFGE has been accepted extensively in epidemiological investigations because it has a high discriminatory power (Arakawa *et al*, 2000; Bhowmick *et al*, 2007).

The objectives of this study were: 1) to examine whether *V. cholerae* isolated from different provinces in the northeastern Thailand carried the same virulence-associated genes (*viz, ctxA, tcpA, zot, ace, ompU, hlyA, stn/sto*) and regulatory gene (*toxR*), 2) to compare the correlation of PFGE patterns of *V. cholerae* isolates from different provinces, and 3) to determine whether these *V. cholerae* isolates had the same patterns of antimicrobial resistance.

MATERIALS AND METHODS

Bacterial strains

A total of 84 *V. cholerae* O1 isolates were collected in 2007-2008 from patients who contracted cholera during an outbreak in Khon Kaen (50 isolates), Udon Thani (23 isolates), Nong Khai (7 isolates) and Loei (4 isolates) Provinces in northeastern Thailand.

Culture technique

V. cholerae O1 isolates were streaked onto thiosulfate-citrate-bile-salt-sucrose (TCBS) agar (Oxoid, Unipath, Basingstroke, Hamshire, UK) and incubated at 37ºC for 24 hours. The yellow colonies were re-streaked on blood agar and confirmed to be *V. cholerae* by standard biochemical tests (Koneman, 1997).

Serotyping

V. cholerae colonies were agglutinated with polyvalent *V. cholerae* O1/O139 antiserum and then categorized by serotype using specific monovalent antiserum (Oxoid, Columbia, MD) against Inaba and Ogawa. *V. cholerae* isolates that did not agglutinate with the polyvalent *V. cholerae* O1/O139 antiserum were classified as *V. cholerae* non-O1/O139.

Reverse passive latex agglutination assay (RPLA)

Latex agglutination assay for detecting cholera toxin was performed according to manufacturer's instructions (Oxoid, Columbia, MD) using a latex agglutination kit (VET-RPLA). In brief, *V. cholerae* was grown in peptone water (pH 8.4) and incubated at 30ºC for 24 hours with shaking. The overnight culture were centrifuged at 900*g* for 20 minutes at 4ºC. Then, a 25 µl aliquot of supernatant was mixed with a 25 µl of the latex suspension in V-well microtiter plates. If toxin is present (positive result), agglutination occurs which results in the formation of a lattice structure.

PCR assay

DNA was extracted using a genomic DNA purification kit (Puregene DNA purification system, Gentra system, Minneapolis, USA) according to the manufacturer's protocol. Specific primers designed for multiplex PCR analysis of *ctxA, tcpA, zot, ace, ompU, stn/sto, hlyA* and *toxR* were employed (Chomvarin *et al*, 2008). PCR was performed in a total volume of 50 µl containing 200 µM of each dNTP (Gibco-BRL, Life Technologies, Gaithersburg, MD), 0.75-1.5 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 100-300 nM of each primer, 100-200 ng of target DNA and 1.25 U *Taq* polymerase (Gibco-BRL). A thermocycling (Gene Amp, PCR 2400, Perkin-Elmer, Doaners Grove, IL) conditions and expected amplicon sizes are summarized in Table 1. Amplicons were subjected to 2% agarose gel-electrophoresis and visualized under UV light (Imagemaster VDS, Pharmacia Biotech, SanDiago, CA) after ethidium bromide staining.

PFGE

Vibrio cholerae O1 isolates were subjected to PFGE as described previously (Arakawa *et al*, 2000). In brief, bacterial cells were embedded in 1% SeaKem Gold agarose (FMC, Lonza, Rockland, ME) and lysed with 1% Sakosyl in 0.5 mM Tris, 0.5 mM EDTA, pH 8.0, containing 0.1 mg/ml protease K. DNA then was digested with 40 U *Not* I (Sib Enzyme, Novosibirsk, Russia) at 37ºC. PFGE was performed in 1% SeaKem Gold agarose in 0.5X Tris-borate-EDTA buffer with a CHEF Mapper system (BioRad Laboratories, Hercules, CA) under the following condition: 14ºC, 6V/cm, field angle of 120º, a linearly ramped switching time of 4 seconds to 8 seconds for 9 hours and for a further 8 to 50 seconds for 11 hours (Arakawa *et al*, 2000). *Salmonella* ser. Braenderup H9812 standard was used as the molecular weight marker.

Cluster analysis of PFGE patterns was conducted using BioNumerics (Version 4.6). Dice coefficient and UPGMA (unweighted pair group method with arithmetic averages) were used to compare the pattern profiles (Arakawa *et al*, 2000).

Antimicrobial susceptibility test

Antimicrobial susceptibility test was performed using standard disk agar diffusion method (NCCLS, 2002; Schroeder *et al*, 2002) with commercially manufactured

Table 1
Primers, amplicon sizes and PCR conditions used for detection of virulence-associated genes of *V. cholerae*.

Gene and amplicon size (bp)	Primer sequence	PCR condition	Reference
ctxA, 302	F-5′ CTCAGACGGGATTTGTTAGGGACG 3′	95ºC, 30 sec; 60ºC,	(Kapley and Purohit, 2001;
	R-5′ TCTATGTCTGTAGCCATT 3′	1 min; 72ºC, 1 min	Mukhopadhyay *et al*, 2001;
tcpA, 472	F-5′ GAAGAAGTTTGTAAAAGAAGAACAC 3′	(25 cycles)	Rivera *et al*, 2001)
	R-5′ GAAAGCACCTTCTTTCAGGTTG 3′		
zot, 947	F-5′ TCGCTTAACGATGGCGCGTTTT 3′		
	F-5′ AACCCCGTTTCACTTCTACCCA-3′		
ace, 600	F-5′ AGAGCGCTGCATTTATCCTTATTG 3′	95ºC, 30 sec; 60ºC,	(Rivera *et al*, 2001; Singh *et al*,
	R-5′ AACTCGGTCTCGGCCTCTCGTATC 3′	1 min; 72ºC, 1 min	2002; Leal *et al*, 2004)
toxR, 779	F-5′ CCTTCGATCCCCTAAGCAATAC 3′	(25 cycles)	
	R-5′ AGGGTTAGCAACGATGCGTAAG 3′		
ompU, 869	F-5′ ACGCTGACGGAATCAACCAAAG 3′		
	R-5′ GCGGAAGTTTGGCTTGAAGTAG 3′		
stn/sto, 140	F-5′ AAAACAGTGCAGCAACCACAAC 3′	95ºC, 30 sec; 55ºC, 1 min;	Modified from
	R-5′ GCTGGATTGCAACATATTTCGC 3′	72ºC, 1 min (25 cycles)	(Mukhopadhyay *et al*, 1995;
hlyA, 540	F-5′ CTTAGCTGAGCTGCGCGATTTG 3′		Rivera *et al*, 2001)
	R-5′ GAGTTGATCATTCAGA 3′		

Table 2
Distribution of virulence and regulatory genes in 84 *V. cholerae* O1 isolates from patients with diarrhea in northeastern Thailand.

Source	Number of isolates	Genotype presence (+) or absence (-) of genes							
		ctxA	*tcpA*	*zot*	*ace*	*toxR*	*ompU*	*stn/sto*	*hlyA*
Khon Kaen	50	+	+	+	+	+	+	-	+
Udon Thani	19	+	+	+	+	+	+	-	+
	4	-	+	-	-	+	+	-	+
Nong Khai	6	+	+	+	+	+	+	-	+
	1	-	+	-	-	+	+	-	+
Loei	4	+	+	+	+	+	+	-	+

disks (Oxoid, Unipath, Basingstroke, Hamshire, England). All *V. cholerae* isolates were examined for resistance to ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), Co-trimoxazole (25 µg), erythromycin (15 µg), norfloxacin (10 µg), and tetracycline (30 µg). Control strain was *E. coli* ATCC 25922. The inhibitory zones were determined as resistant (R), intermidiate resistant (I), and susceptible (S) according to each antimicrobial agent. For the intermidiate resistance, the size of zone is between R and S.

RESULTS

Distribution of virulence-associated genes of *V. cholerae* O1

All 84 *V. cholerae* O1 isolates belonged to El Tor biotype and Ogawa serotype. Using multiplex PCR, all *V. cholerae* O1 strains carried the virulence-associated genes (*ctxA*, *tcpA*, *zot*, *ace*, *ompU*, *hlyA* and *toxR*), except four strains (5%) from Udon Thani and one strain (1%) from Nong Khai, which carried only *tcpA*, *ompU*, *hlyA* and *toxR* (Table 2). None of the *V. cholerae* O1 strains carried *sto* and all carried *hlyA* and *toxR*. All *V. cholerae* strains carrying *ctxA* (94%) produced cholera toxin (as determined by RPLA).

PFGE profile

Based on frequency of isolation, period of time and antibiogram, 16 strains of *V. cholerae* O1 were randomly selected for differentiation using PFGE, which revealed different patterns (A and B) amongst these 16 isolates (Fig 1). A dendrogram presenting the similarity of the 16 *V. cholerae* O1 isolates from the four provinces is also shown.

Antimicrobial susceptibility testing

Antimicrobial susceptibility of the 84 *V. cholerae* O1 isolates was tested against ampicillin (AMP), chloramphenicol (C), ciprofloxacin (CIP), Co-trimoxazole (SXT), erythromycin (E), norfloxacin (NOR) and tetracycline (TE). Twelve antimicrobial susceptibility (AS) types were found and the most frequent antibiogram pattern was AMP^S, C^S, CIP^S, E^I, SXT^R, NOR^S and TE^R, and only one *V. cholerae* isolate was sensitive to all seven various antimicrobials tested (Table 3). Multi-drug resistance to SXT and TE was found in the *V. cholerae* O1 strains isolated from Khon Kaen (48/50) (Table 4), Udon Thani (13/23) and Loei (3/4). All *V. cholerae* O1 isolates were susceptible to NOR, and intermediate resistance to CIP was found only in two strains isolated from patients in Khon

Table 3
Antimicrobial susceptibility (AS) types among 84 *V. cholerae* O1 isolates from patients with diarrhea in northeastern Thailand.

AS type	Antimicrobial agent							KK	UT	NK	LI	Total number (%)
	AMP	C	CIP	E	SXT	NOR	TE					
1	S	S	S	S	S	S	S			1		1 (1)
2	S	S	S	I	S	S	S		1			1 (1)
3	S	S	S	I	I	S	S		1			1 (1)
4	S	S	S	S	R	S	S		1			1 (1)
5	S	S	S	I	R	S	S	2	1	3		6 (7)
6	S	S	S	I	R	S	I		6	1		7 (8)
7	S	S	S	S	R	S	I			2	1	3 (4)
8	S	S	S	I	R	S	R	44	11		1	56 (66)
9	R	S	S	I	R	S	R	2	1		1	4 (5)
10	S	S	S	R	R	S	R		1			1 (1)
11	S	S	I	I	R	S	R	1			1	2 (2)
12	S	R	S	R	S	S	R	1				1 (1)

R, resistant; I, intermediate; S, susceptible; AMP, ampicillin; C, chloramphenicol; CIP, ciprofloxacin; E, erythromycin; SXT, Co-trimoxazole, NOR, norfloxacin; TE, tetracycline; KK, Khon Kaen; UT, Udon Thani; NK, Nong Khai; LI, Loei

Table 4
Antimicrobial resistance of 84 *V. cholerae* O1 isolates from diarrheal patients in northeastern Thailand.

No. of combinations of antimicrobial resistance	Number of *V. cholerae* resistant isolates (%)			
	Khon Kaen	Udon Thani	Nong Khai	Loei
0	0 (0)	2 (9)	1 (14)	0 (0)
1	2 (4)	8 (35)	6 (86)	1 (25)
2	45 (92)	12 (52)	0 (0)	2 (50)
3	3 (6)	2 (9)	0 (0)	1 (25)

Kaen and Loei. The geographical origins of *V. cholerae* isolates resistant to one, two or three antibiotics are shown in Table 4.

DISCUSSION

Understanding the genetic changes and monitoring the virulence genes of pathogenic *V. cholerae* in northeastern Thailand could be useful for controlling new potentially epidemic clones and for understanding the evolution of these microorganisms. We compared 84 *V. cholerae* strains isolated from diarrheal patients in four provinces (Khon Kaen, Udon Thani, Nong Khai and Loei) during the 2007-2008 cholera outbreak.

All *V. cholerae* O1 were of the Ogawa serotype. All strains isolated from Khon Kaen and Loei Provinces carried several

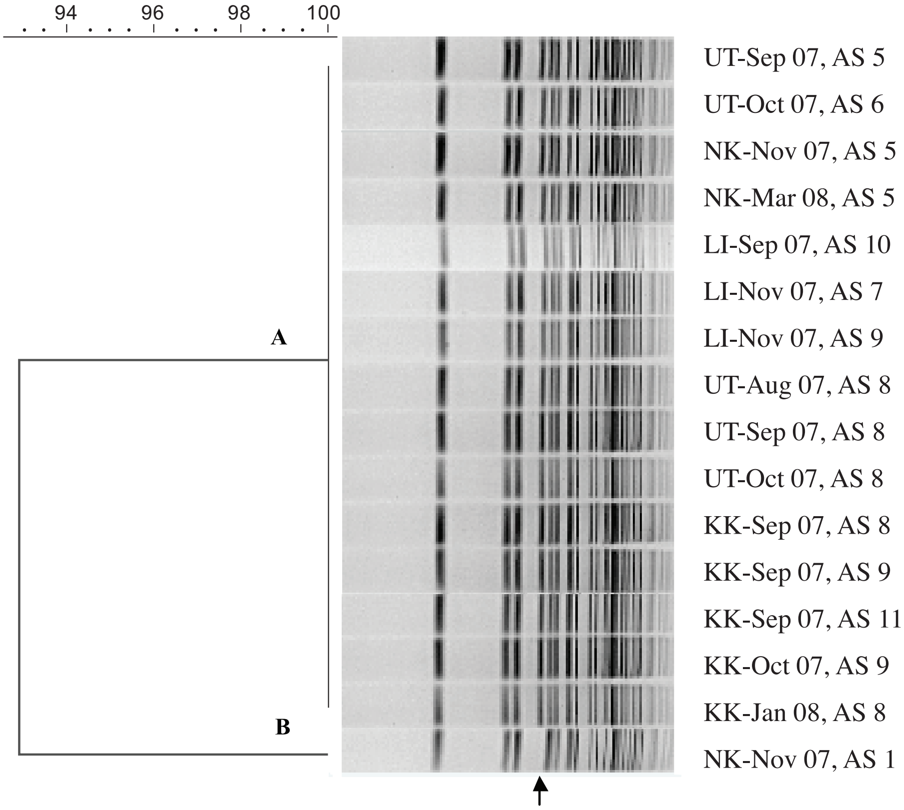

Fig 1–PFGE patterns and dendrogram showing the relatedness of *V. cholerae* O1 isolates. KK, Khon Kaen; UT, Udon Thani; NK, Nong Khai; LI, Loei. AS, antimicrobial susceptibility type as indicated in Table 3. The month and year indicates the various time of isolation. Arrow indicates polymorphic band.

virulence-associated genes (including *ctxA, tcpA, zot, ace, ompU, hlyA* and *toxR*, except *sto*) while four isolates and one isolate from Udon Thani and Nong Khai, respectively, were positive for *tcpA, ompU, hlyA* and *toxR* but not for *ctxA*, *zot*, *ace* and *sto*. These results suggest that the majority of *V. cholerae* O1 strains found in this area were pathogenic, which was confirmed by RPLA agglutination test. The 5 isolates not carrying *ctx* did not produce cholera toxin. This may reflect deletion of the toxin gene in the virulence cassette (Damian *et al*, 1998; Pourshafie *et al*, 2002). Generally, *V. cholerae* non-O1/non-O139 has been called "non-toxigenic" (NT) *V. cholerae*, while *V. cholerae* O1/O139 is toxigenic because it carries *ctx*A, *zot* and *ace* (Saha *et al*, 1996; Tapchaisri *et al*, 2007). However, we found that *ctx*A, *zot*, and *ace* could be missing, but that the isolates still had the ability to cause severe diarrhea. This may be due to other virulence factors and other mechanisms involved in the pathogenesis of these strains, such as presence of *hlyA* encoding El Tor hemolysin or cytolysin, which can induce apoptosis (Saka *et al*, 2007) and cause cell vacuolation and death (Morin *et al*, 2004).

PFGE has been accepted as the standard method for genetic diversity analysis of bacterial species, including *V. cholerae* (Makino *et al*, 1995; Radu *et al*, 1999). In this study, PFGE was able to differentiate randomly selected *V. cholerae* O1 into two groups. PFGE type A (15/16) was prevalent in all four provinces (Khon Kaen, Udon Thani, Nong Khai and Loei), suggesting that these drug-resistant strains may have derived from a common clone. The differences in their antibiograms might be due to mutations in the genes associated with resistance to the antimicrobial agents, although they appear to have derived from the same clone. Only one isolate from Nong Khai was PFGE type B and was sensitive to all seven antimicrobial agents, suggesting this strain may be derived from a different clone, which was a very closely clone, because it showed more than 90% similarity to PFGE type A.

The majority of *V. cholerae* O1 isolates from Khon Kaen, Udon Thani and Loei (but notably not from Nong Khai) were multi-drug resistant. *V. cholerae* strains resistant to SXT and TE have emerged predominantly in Khon Kaen, and strains isolated from patients in this province were more resistant to antimicrobial agents than isolates from the other provinces, although all *V. cholerae* O1 isolates from all four provinces were susceptible to NOR. However, an intermediate resistance to CIP was found in two strains from Khon Kaen and Loei. The types of antimicrobial resistance emerged could reflect exposure of these strains to the antimicrobial agents selected for use in each region. Thus, the importance of the appropriate use of antimicrobial agents is vital in order to prevent the further spread of antimicrobial resistance.

In summary, our results demonstrated that all 84 *V. cholerae* O1 strains isolated from patients in four provinces in northeastern Thailand carry virulence-associated genes (except *sto*). General surveillance of *V. cholerae* O1 should include monitoring of the genotypes of virulence-associated genes, PFGE profiles and antibiograms; in order to have a thorough epidemiological basis for detecting and coping with future emergence and outbreak of multidrug resistant *V. cholerae*.

ACKNOWLEDGEMENTS

This study was supported by research grants from the Faculty of Medicine, Khon Kaen University, Thailand. We thank the staff of the Office of Communicable Disease Control, Region 6, Khon Kaen for their help with specimen collection, the National Institute of Public Health and Srinagarind Hospital, Thailand for providing bacterial reference strains.

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