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Drug response and genetic properties of *Vibrio cholerae* associated with endemic cholera in north-eastern Thailand, 2003–2011

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Cholera, caused by *Vibrio cholerae*, results in significant morbidity and mortality worldwide, including Thailand. Representative *V. cholerae* strains associated with endemic cholera (n=32), including strains (n=3) from surface water sources, in Khon Kaen, Thailand (2003–2011), were subjected to microbiological, molecular and phylogenetic analyses. According to phenotypic and related genetic data, all tested *V. cholerae* strains belonged to serogroup O1, biotype El Tor (ET), Inaba (IN) or Ogawa (OG). All of the strains were sensitive to gentamicin and ciprofloxacin, while multidrug-resistant (MDR) strains showing resistance to erythromycin, tetracycline, trimethoprim/sulfamethoxazole and ampicillin were predominant in 2007. *V. cholerae strains* isolated before and after 2007 were non-MDR. All except six diarrhoeal strains possessed *ctxA* and *ctxB* genes and were toxigenic altered ET, confirmed by MAMA-PCR and DNA sequencing. Year-wise data revealed that *V. cholerae* INET strains isolated between 2003 and 2004, plus one strain isolated in 2007, lacked the RS1 sequence (*rstC*) and toxin-linked cryptic plasmid (TLC)-specific genetic marker, but possessed CTX^{CL} prophage genes *ctxB*^{CL} and *rstR*^{CL}. A sharp genetic transition was noted, namely the majority of *V. cholerae* strains in 2007 and all in 2010 and 2011 were not repressor genotype *rstR*^{CL} but instead were *rstR*^{ET}, and all *ctx*$^{+}$ strains possessed RS1 and TLC-specific genetic markers. DNA sequencing data revealed that strains isolated since 2007 had a mutation in the *tcpA* gene at amino acid position 64 (N→S). Four clonal types, mostly of environmental origin, including subtypes, reflected genetic diversity, while distinct signatures were observed for clonally related, altered ET from Thailand, Vietnam and Bangladesh, confirmed by distinct subclustering patterns observed in the PFGE (*Not*I)-based dendrogram, suggesting that endemic cholera is caused by *V. cholerae* indigenous to Khon Kaen.

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Abbreviations: AMP, ampicillin; CL, classical; CTX, cholera toxin; E, erythromycin; ET, El Tor; MAMA, mismatch amplification mutation assay; MDR, multidrug resistant; SXT, trimethoprim/sulfamethoxazole; TE, tetracycline; TLC, toxin-linked cryptic plasmid.

INTRODUCTION

Cholera is a severe form of acute diarrhoea, caused by the gamma-proteobacterium *Vibrio cholerae*. *V. cholerae* comprises more than 200 'O' serogroups even though the disease historically has been considered to be caused only by *V. cholerae* serogroups O1 and O139 (Alam *et al.*, 2007). Serogroup O1 has two biotypes, Classical (CL) and El Tor (ET), of which the former is associated with the first six pandemics starting in 1817 before the latter was isolated at the beginning of the current ongoing seventh cholera pandemic in the 1960s (Kaper *et al.*, 1995). The CL biotype differs from the ET biotype in some phenotypic traits, primarily haemolysis of sheep erythrocytes, agglutination of chicken erythrocytes, Voges–Proskauer reaction, sensitivity to polymyxin B and sensitivity to specific phages (Kaper *et al.*, 1995). Additionally, genotypic tests have been used to determine biotypes of *V. cholerae*, such as presence of *tcpA* (encoding toxin co-regulated pilin A), *ctxB* (cholera toxin B), *rstR* (repeat sequence transcriptional regulator) and presence or absence of *rtxC* (repeat in the toxin gene), and epitope analysis of one of the two subunits of cholera toxin (CTB epityping) has been performed (Safa *et al.*, 2010). The VSP-I and -II gene clusters are unique to ET strains of the seventh pandemic, in that they are absent from both the pre-seventh pandemic ET strains and CL biotype strains (Dziejman *et al.*, 2002). The seventh and current cholera pandemic is ascribed to the ET biotype, while the fifth and sixth pandemics are associated with the CL biotype (Alam *et al.*, 2010).

A significant and relatively recent development is the emergence of altered ET *V. cholerae* strains harbouring cholera toxin (CTX) and certain related traits of the CL biotype, which was first isolated in Asia in 2001, displacing the prototype ET in frequency of isolation from its Asian habitats (Nair *et al.*, 2006). The ET biotype associated with cholera in Africa in the 1970s was similarly replaced by an altered ET (Morita *et al.*, 2010). Results of genetic analysis revealed that these different ET strains vary in type of CTX prophage and flanking RS1 elements in the genome of the toxigenic strains. *V. cholerae* altered ET strains in Africa were shown to be different from those in Asia since they harboured the entire CL CTX prophage in an ET biotype background (Ansaruzzaman *et al.*, 2004). According to a recent report, altered ET strains were predominant among CL and ET biotype progenitors associated with endemic cholera in Mexico between 1991 and 1997 (Alam *et al.*, 2010). Such altered ET strains were found to cause a more severe disease in Asia (Siddique *et al.*, 2010), and are being reported globally (Chin *et al.*, 2011; Na-Ubol *et al.*, 2011; Okada *et al.*, 2010; Goel *et al.*, 2011).

Cholera is endemic and a major public health concern in Thailand (Bureau of Epidemiology, 2010), especially for lower socio-economic groups in the north-east region (Tangkanakul & Hanpanjakit, 2007). In Thailand, endemic cholera causes significant morbidity and mortality each year. For example, 986 cases of cholera were reported by the Department of Disease Control, Ministry of Public Health of Thailand in 2007, of which seven cases were fatal (Bureau of Epidemiology, 2010). At the beginning of 2010, outbreaks of cholera were reported in 15 provinces, including Khon Kaen, the largest provincial city in north-east Thailand. Although treatment for cholera includes a 3 day course of effective antibiotics and rehydration therapy, a progressive increase in drug resistance makes cholera treatment very difficult, not only in Thailand but worldwide (Ang *et al.*, 2010; Jain *et al.*, 2011; Kumar *et al.*, 2010a; Quilici *et al.*, 2010). A recent study reported limited phenotypic, genotypic and virulence characteristics of *V. cholerae* O1 strains associated with endemic cholera in Thailand (Na-Ubol *et al.*, 2011; Okada *et al.*, 2012). However, the study did not address the antibiotic response of *V. cholerae* or the source of cholera. To understand drug response and molecular and phylogenetic trends of *V. cholerae* associated with endemic cholera in north-eastern Thailand, representative *V. cholerae* strains (n=35) were isolated between 2003 and 2011 from diarrhoea patients (n=32) and environmental (n=3) sources in Khon Kaen and all isolates were subjected to microbiological, molecular and phylogenetic analyses.

METHODS

Bacterial strains. A total of 35 *V. cholerae* O1 strains, 32 of which were associated with cholera and 3 from natural surface water sources in Khon Kaen, Thailand, between 2003 and 2011, were examined for phenotypic characteristics, namely antimicrobial response and virulence, and for molecular traits, including phylogenetic characteristics. *V. cholerae* O1 strains N16961 (ET biotype) and O395 (CL biotype) served as controls. All were cultured overnight on selective bacteriological media, including taurocholate tellurite gelatin (TTGA) and thiosulfate citrate bile salts sucrose agar. *V. cholerae* colonies were confirmed using a combination of biochemical, serological and molecular methods, as described previously (Alam *et al.*, 2007).

Antibiotic susceptibility. Susceptibility to antibiotics was determined by disc diffusion, as described by Bauer *et al.* (1966) and the Clinical and Laboratory Standards Institute (CLSI, 2010), using commercial antibiotic discs. Six antibiotics (Oxoid) were employed: erythromycin (15 μg), gentamicin (10 μg), trimethoprim/sulfamethoxazole (30 μg), tetracycline (30 μg), ampicillin (30 μg) and ciprofloxacin (5 μg). The resistance or susceptibility profiles of the isolates were determined by measuring the inhibitory zone and comparing it with an interpretative chart to determine sensitivity to the antibiotics.

Serogroup and biotype. Serogroups of the *V. cholerae* isolates identified using biochemical and molecular methods were confirmed by slide agglutination using specific polyvalent antisera for *V. cholerae* O1 and O139, followed by screening with a monoclonal antibody specific for each serogroup (Alam *et al.*, 2007). Biotyping primarily involved selective phenotypic tests, including chicken erythrocyte agglutination, and sensitivity to polymyxin B, Mukherjee CL phage IV and Mukherjee ET phage V (Kaper *et al.*, 1995). Serogrouping and biotyping were further confirmed by PCR as described below.

Genomic DNA preparation. Genomic DNA extraction was done by described methods (Nusrin *et al.*, 2009).

PCR assays for serogroup and biotype determination. Subtypes of all strains were reconfirmed using *V. cholerae* species-specific *omp*W PCR (Nandi *et al.*, 2000). Serogroups were reconfirmed using multiplex PCR targeted at O1- (*wbeO1*) and O139- (*wbfO139*) specific O biosynthetic genes and the CTX gene (*ctx*A) (Hoshino *et al.*, 1998). Biotype-specific characteristics were determined using PCR assays targeted to *tcpA* (CL and ET) (Rivera *et al.*, 2001), *rstR*, encoding phage transcriptional regulator (Mwansa *et al.*, 2007), presence of the repeat in toxin (*rtxC*) (Chow *et al.*, 2001), *rstC*, encoding an anti-repressor protein, and *tlc*, encoding the toxin-linked cryptic plasmid (O'Shea *et al.*, 2004).

Primers used in this study are shown in Table 1.

Determination of *ctxB* genotype by MAMA-PCR. The mismatch amplification mutation assay (MAMA) was recently developed to detect sequence polymorphism between the CL and ET *ctxB* genes (*ctxB*^{CL} and *ctxB*^{ET}, respectively) by focusing on nucleotide position 203 of the *ctxB* gene (Morita *et al.*, 2008). MAMA-PCR was used to test for presence of *ctxB* specific for CL and ET biotypes. A conserved forward primer (Fw-con, 5′-ACTATCTTCAGCATATGCACATGG-3′) and two allele-specific polymorphism detection primers, Rv-cl (5′-CCTGGTA-CTTCTACTTGAAACG-3′) and Rv-et (5′-CCTGGTACTTCTACT-TGAAACA-3′), were used. PCR conditions were as follows: initial denaturation at 96 °C for 2 min, 25 cycles of denaturation at 96 °C for 10 s, annealing at 50 °C for 10 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 2 min. *V. cholerae* O1 CL O395 and ET N16961 served as reference strains.

Nucleotide sequencing of *ctxB* and *tcpA*. The *ctxB* and *tcpA* genes of representative *V. cholerae* O1 strains from each year (2003–2011) were sequenced following conditions described elsewhere (Olsvik *et al.*, 1993). PCR amplification of *ctxB* and *tcpA* was performed in a 25 µl reaction mixture in an automated Peltier thermal cycler (PTC-200, M. J. Research). PCR products were purified with a Microcon centrifugal filter device (Millipore) and sequenced using an ABI PRISM Big Dye Terminator Cycle Sequencing Reaction kit (Applied Biosystems) on an ABI PRISM 310 automated sequencer (Applied Biosystems). The sequences of the respective genes for other *V. cholerae* O1 ET and CL strains listed in Fig. 1 were retrieved from GenBank (accession numbers NC_002505, U25679, EU496278). The deduced amino acid sequences of the respective genes from all strains were aligned using CLUSTAL W.

PFGE. Whole agarose-embedded genomic DNA from the *V. cholerae* isolates was prepared. PFGE was carried out using a contour-clamped homogeneous electrical field (CHEF-DRII) apparatus (Bio-Rad), according to procedures described elsewhere (Cameron *et al.*, 1994). Conditions for separation were as follows: 2 to 10 s for 13 h, followed by 20 to 25 s for 6 h. An electrical field of 6 V cm^{-1} was applied at an included field angle of 120°. Genomic DNA of the test strains was

Table 1. PCR primers used in this study

Primer	Sequence (5′–3′)	Targeted gene	Annealing temp. (°C)	Amplicon size (bp)	Reference
ompW F	CAC CAA GAA GGT GAC TTT ATT GTG	*ompW*	64	304	Nandi *et al.* (2000)
ompW R	GGT TTG TCG AAT TAG CTT CAC C				
rfbO1 F	GTT TCA CTG AAC AGA TGG G	*rfbO1*	55	192	Hoshino *et al.* (1998)
rfbO1 R	GGT CAT CTG TAA GTA CAA C				
rfbO139 F	AGC CTC TTT ATT ACG GGT GG	*rfbO139*	55	449	Hoshino *et al.* (1998)
rfbO139 R	GTC AAA CCC GAT CGT AAA GG				
ctxA F	ACA GAG TGA GTA CTT TGA CC	*ctxA*	55	308	Hoshino *et al.* (1998)
ctxA R	ATA CCA TCC ATA TAT TTG GGA G				
tcpA ET R	CGA AAG CAC CTT CTT TCA CAC GTT G	*tcpA* ET	60	453	Rivera *et al.* (2001)
tcpA F	CAC GAT AAG AAA ACC GGT CAA GAG				
tcpA class R	TTA CCA AAT GCA ACG CCG AAT G	*tcpA* CL	60	620	Rivera *et al.* (2001)
tcpA F	CAC GAT AAG AAA ACC GGT CAA GAG				
MAMA ElTor F	ACT ATC TTC AGC ATA TGC ACA TGG	*ctxB* ET	55	186	Morita *et al.* (2008)
MAMA ElTor R	CCT GGT ACT TCT ACT TGA AAC A				
MAMA class F	ACT ATC TTC AGC ATA TGC ACA TGG	*ctxB* CL	55	186	Morita *et al.* (2008)
MAMA class R	CCT GGT ACT TCT ACT TGA AAC G				
rstR1 F	CTT CTC ATC AGC AAA GCC TCC ATC	*rstR* CL	50	500	Mwansa *et al.* (2007)
rstR3A R	TCG AGT TGT AAT TCA TCA AGA GTG				
rstR2 F	GCA CCA TGA TTT AAG ATG CTC	*rstR* ET	50	500	Mwansa *et al.* (2007)
rstR3A R	TCG AGT TGT AAT TCA TCA AGA GTG				
rtxC F	CGA CGA AGA TCA TTG ACG AC	*rtx*C	55/56	265	Chow *et al.* (2001)
rtxC R	CAT CGT CGT TAT GTG GTT GC				
rstC1	AAC AGC TAC GGG CTT ATT C	*rstC*	52.4	238	O'Shea *et al.* (2004)
rstC2	TGA GTT GCG GAT TTA GGC				
tlc3	GGG AAT GTT GAG TTC TCA GTG	*tlc*	55.5	1548	O'Shea *et al.* (2004)
tlc4	GTT GCG AAG TGG ATT TTG TG				
tcpA-F	ATG CAA TTA TTA AAA CAG CTT TTT AAG	*tcpA*	59	675	Kumar *et al.* (2010b)
tcpA-R	TTA GCT GTT ACC AAA TGC AAC AG				
CTX7	GGT TGC TTC TCA TCA TCG AAC CAC	*ctxB*	55	460	Olsvik *et al.* (1993)
CTX9B	GAT ACA CAT AAT AGA ATT AAG GAT				

digested using *Not*I (Gibco-BRL), and *Salmonella enterica* serovar Braenderup was digested using *Xba*I, with fragments employed as molecular size markers. Restriction fragments were separated in 1 % pulsed-field-certified agarose in 0.5× TBE (Tris/borate/EDTA) buffer. Post-electrophoresis gel treatment included gel staining and de-staining. The DNA was visualized using a UV transilluminator, and images were digitized via a one-dimensional gel documentation system (Bio-Rad).

Image analysis. The fingerprint pattern in the gel was analysed using the Bionumeric software (Applied Maths). After background subtraction and gel normalization, the fingerprint patterns were subjected to typing on the basis of banding similarity and dissimilarity, using the Dice similarity coefficient and unweighted-pair group method employing average linkage (UPGMA) clustering, as recommended by the manufacturer. The results were graphically represented as dendrograms.

RESULTS

Microbiology and serology

The *V. cholerae* strains included in the present study are shown in Table 2, with source and year of isolation. All *V. cholerae* O1 strains [clinical patient (n=32) and environmental (n=3) isolates] included in this study produced colonies typical of *V. cholerae* on TTGA and gave biochemical reactions characteristic for *V. cholera*e. All reacted positively to *V. cholerae* O1 monoclonal antibody, but not to O139. They also were positive for either Inaba or Ogawa serotype-specific monovalent antisera, confirming that all belonged to serogroup O1.

Serotyping results showed that both environmental and clinical strains of *V. cholerae* isolated between 2003 and 2004 were Inaba. All *V. cholerae* O1 clinical strains isolated in 2007 and 2011 and a majority of clinical strains isolated in 2010 were Ogawa. Two strains isolated from surface water sources in 2010 were Inaba (Table 2).

Amplification of primers specific for *V. cholerae* serogroup O1 and *ctxA* by PCR

All strains amplified primers for *V. cholerae* species-specific *ompW* and all amplified primers specific for the O biosynthetic gene *wbe* of *V. cholerae* O1, but not *wbf*, which is specific for serogroup O139. In addition, all except five clinical strains associated with cholera during 2010, plus one strain isolated in 2011, amplified primers for the CTX gene *ctxA*, confirming that the strains were toxigenic *V. cholerae* O1. Six *V. cholerae* O1 strains that did not amplify primers for *ctxA* were concluded to be *ctx* negative (Table 2).

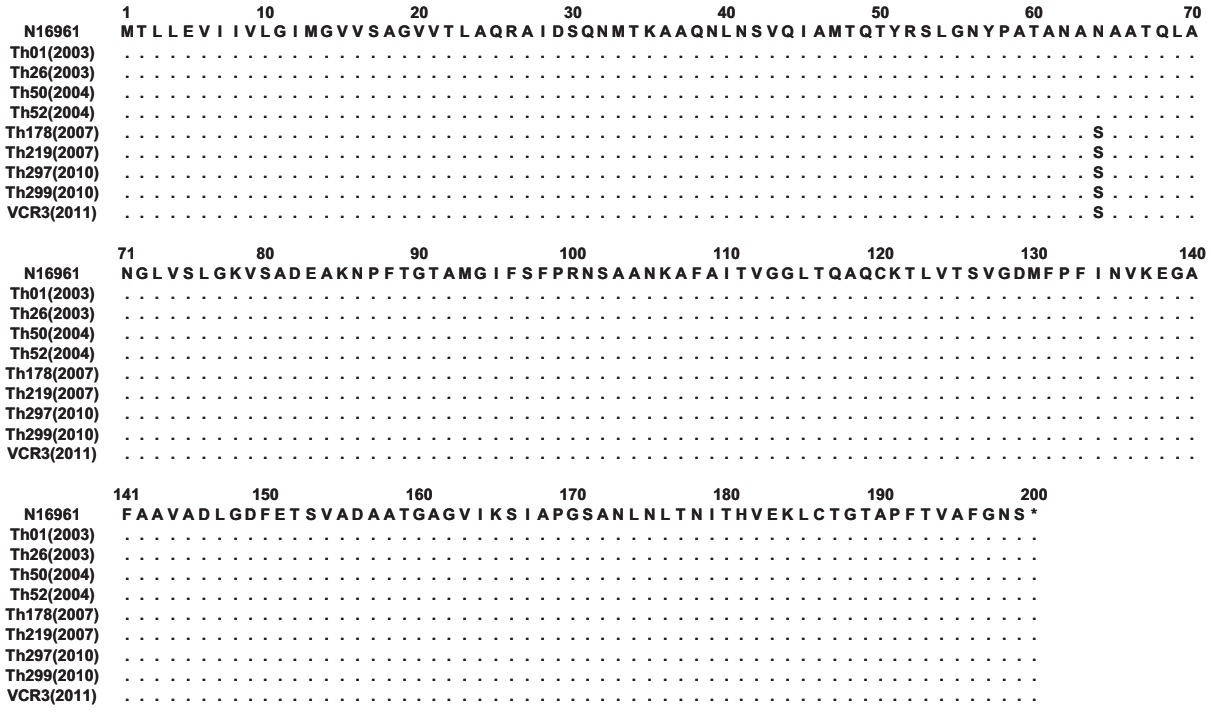

Fig. 1. CLUSTAL W alignment of amino acids encoded by the *tcpA* gene of the test *V. cholerae* O1 strains with the sequence from the ET reference strain N16961. Dots indicate identical amino acids. The amino acid sequences of TcpA of *V. cholerae* O1 strains isolated in Thailand between 2003 and 2004 showed 100 % identity with those of TcpA of reference strain N16961. Strains isolated in 2007 and thereafter showed a mutation at amino acid position 64 (N→S) of the *tcpA* gene.

Table 2. Phenotypic, genotypic and drug resistance properties of *V. cholerae* O1 isolated in Thailand (n=35) between 2003 and 2011

Strains	Year of isolation	No. of isolates	Source*	Serotype	*rfbO1*	Phenotypic properties†				Genetic screening by PCR							Resistance pattern§
						CCA	Sensitivity			*ctxA*	*tcpA* type	*ctxB* type‡	*rstR* type	*rtxC*	*rstC*	*tlc*	
							PMB (50 U)	CL-specific phage IV	ET-specific phage V								
Thai isolates	2003	1	Clin	Inaba	+	+	R	R	S	+	ET	CL	CL	+	−	−	E, AMP
	2003	1	Env	Inaba	+	+	R	R	S	+	ET	CL	CL	+	−	−	E, AMP
	2003	1	Clin	Inaba	+	+	R	R	S	+	ET	CL	CL	+	−	−	E
	2003	2	Clin	Inaba	+	+	R	R	S	+	ET	CL	CL	+	−	−	E
	2004	1	Clin	Inaba	+	+	R	R	S	+	ET	CL	CL	+	−	−	E, AMP
	2004	1	Clin	Inaba	+	+	R	R	S	+	ET	CL	CL	+	−	−	E
	2004	1	Clin	Inaba	+	+	R	R	S	+	ET	CL	CL	+	−	−	E
	2007	1	Clin	Ogawa	+	+	R	R	S	+	ET	CL	CL	+	−	−	E, TE, SXT, AMP
	2007	2	Clin	Ogawa	+	+	R	R	S	+	ET	CL	ET	+	+	+	E, TE, SXT, AMP
	2007	2	Clin	Ogawa	+	+	R	R	S	+	ET	CL	ET	+	+	+	E, TE, SXT
	2010	1	Clin	Ogawa	+	+	R	R	S	+	ET	CL	ET	+	+	+	TE, SXT
	2010	1	Clin	Ogawa	+	+	R	R	S	+	ET	CL	ET	+	+	+	E
	2010	1	Clin	Ogawa	+	+	R	R	S	+	ET	CL	ET	+	+	+	TE, SXT
	2010	3	Clin	Inaba	+	+	R	R	S	+	ET	CL	ET	+	+	+	E, SXT
	2010	3	Clin	Inaba	+	+	R	R	S	+	ET	CL	ET	+	+	+	SXT
	2010	1	Clin	Inaba	+	+	R	R	S	−	ET	−	−	+	−	+	E
	2010	1	Clin	Ogawa	+	+	R	R	S	+	ET	CL	ET	+	+	+	E, TE, SXT
	2010	2	Clin	Ogawa	+	+	R	R	S	−	ET	−	−	+	−	+	E, AMP
	2010	1	Clin	Ogawa	+	+	R	R	S	−	ET	−	−	+	−	+	Sensitive to all
	2010	1	Clin	Ogawa	+	+	R	R	S	+	ET	CL	ET	+	+	+	SXT
	2010	1	Clin	Inaba	+	+	R	R	S	+	ET	CL	ET	+	+	+	SXT
	2010	1	Clin	Ogawa	+	+	R	R	S	−	ET	−	−	+	−	+	Sensitive to all
	2010	2	Env	Inaba	+	+	R	R	S	+	ET	CL	ET	+	+	+	E
	2010	1	Clin	Inaba	+	+	R	R	S	+	ET	CL	ET	+	+	+	E, AMP, SXT
	2011	1	Clin	Ogawa	+	+	R	R	S	+	ET	CL	ET	+	+	+	AMP, SXT
	2011	1	Clin	Ogawa	+	+	R	R	S	−	−	−	−	+	−	−	E, AMP, SXT
Reference strains																	
O395	1965	–	Clin	Ogawa	+	−	S	S	R	+	CL	CL	CL	−	−	+	–
N16961	1971	–	Clin	Inaba	+	+	R	R	S	+	ET	ET	ET	+	+	+	–

*Clin, Clinical; Env, environmental.

†CCA, Chicken cell agglutination; PMB, polymyxin B; R, resistant; S, sensitive.

‡Determined by MAMA-PCR.

§AMP, ampicillin; E, erythromycin; SXT, trimethoprim/sulfamethoxazole; TE, tetracycline.

Phenotypic and genetic characteristics

All *V. cholerae* strains showed biotype ET-specific phenotypic traits, such as chicken cell agglutination, sensitivity to ET-specific phage V, and resistance to both polymyxin B and CL-specific phage IV, and were recognized to be biotype ET (Table 2). All, except six *ctx*$^-$ strains, were phenotypic variants of ET with major ET traits, but failed to show all properties typical of *V. cholerae* ET reference strain N16961.

Phenotypically confirmed ET biotype strains amplified primers for the ET-specific marker gene *rtxC*, confirming their identification as *V. cholerae* ET. *tcpA*, the major virulence-associated gene of the VPI-I gene cluster, was present in all strains except the *ctxA*$^-$ strain isolated in 2011, and all amplified primers for *tcpA*^{ET}, but not *tcpA*^{CL}. The *V. cholerae* strains were analysed by MAMA-PCR using primers specific for CL or ET biotype, offering a precise and accurate method for determining the type of CTX. All *V. cholerae* O1 *ctxA*$^+$ strains, including the *V. cholerae* O395 CL reference, amplified primers specific for *ctxB1*^{CL}, an allele identified in CL biotype strains worldwide, including altered ET biotype strains from the US Gulf Coast. Among the toxigenic altered ET strains confirmed in this study, *V. cholerae* isolated from clinical sources and natural surface water between 2003 and 2004, plus a clinical strain isolated in 2007, carried the CL-biotype-specific repressor gene *rstR*^{CL}, but not the ET-biotype-specific repressor gene *rstR*^{ET}, confirming that they carried CL-biotype CTX prophage. However, all of the atypical ET strains isolated in 2003–2004, plus one strain isolated in 2007, were devoid of *rstC* and *tlc*, suggesting a unique genetic characteristic of *V. cholerae* associated with endemic cholera in Thailand. These *V. cholerae* showed a sharp genetic transition, as a majority of the 2007 strains, and all strains (*ctx*$^+$) isolated in Khon Kaen thereafter, until 2011, possessed *rstC* and *tlc* and all carried the repressor *rstR*^{ET}, although the *ctxB* gene of these strains was CL biotype, as was found in altered ET of contemporary cholera (Table 2). The six *ctx*$^-$ *V. cholerae* strains confirmed in this study also lacked *ctxAB*, *rstR* and *rstC*, suggesting that they did not carry the CTX prophage.

Antibiotic susceptibility assay

Antibiotic susceptibility patterns showed that among the 35 *V. cholerae* O1 strains isolated between 2003 and 2011, 71 % (n=25) were resistant to erythromycin, 54 % (n=19) to trimethoprim/sulfamethxazole, 23 % (n=8) to tetracycline, and 31 % (n=11) to ampicillin, whereas all were uniformly sensitive to gentamicin and ciprofloxacin. Overall, 23 % (n=8) of the strains were multidrug resistant (MDR). Year-wise data analysis revealed that, in Thailand, *V. cholerae* showing resistance to four drugs, erythromycin, tetracycline, trimethoprim/sulfamethxazole and ampicillin, occurred among strains isolated in 2007 (Table 2). The majority of *V. cholerae* isolated before or after 2007 were sensitive, although strains varied in their patterns of response to the different drugs tested. Interestingly, two isolates from 2010 were sensitive to all six antibiotics tested (Table 2). Of the eight resistant strains showing resistance to at least three antibiotics, five were isolated in 2007, two in 2010, and only one in 2011. Overall, 54 % of the *V. cholerae* isolated between 2003 and 2011 were resistant to two or more antibiotics.

Sequencing of *ctxB* and *tcpA*

PCR-amplified genes *ctxB* (460 bp) and *tcpA* (675 bp) of randomly selected *V. cholerae* O1 strains (n=9) representing each year between 2003 and 2011 were sequenced and the amino acid sequences were determined by employing bioinformatics tools. The results showed that the deduced amino acid sequence of CTXB of all the tested *V. cholerae* O1 strains was identical to that of the CL biotype CT (*ctxB* genotype 1), with histidine and threonine at positions 39 and 68, respectively. However, the strains isolated since 2007 had a mutation in the *tcpA* gene that resulted an amino acid substitution at position 64 (N→S) in the mature peptide of TcpA (Fig. 1).

PFGE and cluster analysis

The *Not*I-digested genomic DNAs of *V. cholerae* O1 strains from Thailand subjected to PFGE to determine genetic relatedness and clonal origin yielded 20 to 23 fragments (Fig. 2) and their molecular size ranged from 20.5 to 350 kb. All showed ET biotype PFGE patterns, with divergence in the number and position of the DNA fragments. Four major PFGE types, A–D, designated clonal types, and subtypes were determined from the overall PFGE patterns of the 35 *V. cholerae* O1 environmental and clinical strains (Table 2). Clonal type A, with seven different PFGE patterns (A1–A7), was predominantly associated with cholera in Khon Kaen between 2003 and 2011, while clonal type B was represented by a single strain associated with cholera in 2011; clonal types C and D were environmental isolates from surface water samples collected in 2010.

Only the environmental *V. cholerae* strain isolated in 2003 had a PFGE pattern common to clinical strains isolated in 2003 and 2004 (Fig. 2). Year-wise data revealed that all except three *V. cholerae* O1 strains isolated between 2003 and 2011 exhibited closely related PFGE patterns belonging to clonal type A (Fig. 2). The remaining three *V. cholerae* O1 strains, of which two had been isolated in 2010 from surface water and one from a clinical case in 2011, represented three additional clonal types (B–D) from Thailand.

In order to understand the clonal link between the *V. cholerae* O1 strains occurring in Khon Kaen, cluster analysis was performed using PFGE (*Not*I) images of genomic DNA of O1 strains isolated in Thailand (2003–2011), together with representative *V. cholerae* O1 isolates from Bangladesh (2009–2010) and Vietnam (2008–2010). Cluster analysis separated the distinct clonal types and the subtypes although the majority of Thai *V. cholerae* O1 strains constituted a

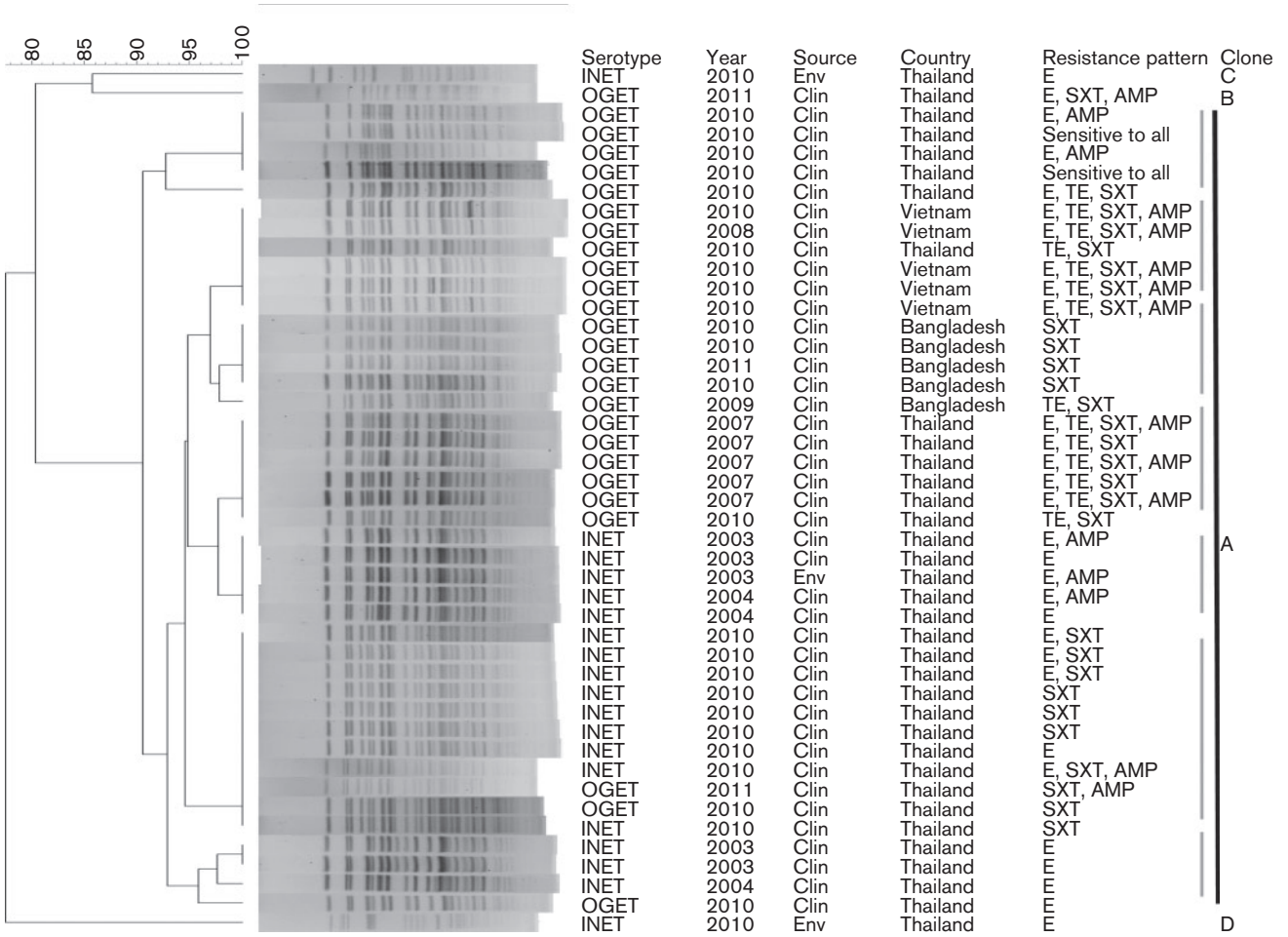

Fig. 2. Genomic fingerprinting patterns of *V. cholerae* O1 strains isolated from diarrhoea and environmental sources in Khon Kaen, Thailand (2003–2011). The dendrogram was prepared by Dice similarity coefficient and UPGMA clustering using PFGE images of the *Not*I-digested genomic DNA. The scale-bar at the top left indicates similarity coefficient (%). The PFGE types (A–D) of the *V. cholerae* O1 strains are shown. The major cluster, A, comprised most of the Thai *V. cholerae* O1 strains (2003–2011) and all of the representative Bangladeshi (2009–2010) and Vietnamese (2008–2010) *V. cholerae* O1 strains showing clonal relatedness. The seven different PFGE pattern-based subclusters within cluster A are marked with grey bars. Distinct signatures for clonally related altered ET in Thailand, Vietnam, and Bangladesh, as confirmed by subclustering patterns in the dendrogram, suggest endemic cholera to be caused by *V. cholerae* population indigenous to Khon Kaen, Thailand. INET, Inaba El Tor; OGET, Ogawa El Tor; Clin, clinical; Env, environmental.

major cluster (A) with all Bangladesh and Vietnam *V. cholerae* O1 isolates, suggesting clonality. None of the contemporary Bangladesh or Vietnam *V. cholerae* O1 strains belonged to clonal types B–D identified in Thailand, suggesting that they were distant clonally (Fig. 2). Although strains of cluster A were deemed 'clonal', based on similarity index, the minor, but consistent, divergence indicated seven subclusters, depending on serotypes (Inaba/Ogawa) and spatio-temporal origin. Country-specific subclustering of the recent Thai, Vietnam and Bangladesh *V. cholerae* O1 isolates reflected their different signatures.

DISCUSSION

V. cholerae causes both epidemic and pandemic cholera and is a serious public health threat for low socio-economic groups in many countries, including Thailand. Research on *V. cholerae* at the molecular level has made enormous strides over the past 100 years (Chun *et al.*, 2009; Kaper *et al.*, 1995). A recent MLVA/PFGE study of *V. cholerae* O1 isolated in Thailand suggested that some clones with similar but distinctive genetic traits circulated during an outbreak (Okada *et al.*, 2012). The present study provides data on antibiotic resistance patterns with

evidence of genetic changes in *V. cholerae* O1 in endemic cholera, namely four major clones with distinct regional signatures in natural surface waters in Khon Kaen, Thailand.

Overall, the results of microbiological, biochemical and serological tests confirmed that 35 *V. cholerae* isolates from cholera outbreaks in north-eastern Thailand between 2003 and 2011 were serogroup O1. Year-wise phenotypic results revealed that *V. cholerae* O1 isolated between 2003 and 2011 showed temporal variation in serotype, Inaba or Ogawa, according to year of isolation. The microbiological results were complemented with simplex PCR assay for amplification of *V. cholerae* species-specific *ompW* (Nandi *et al.*, 2000), together with multiplex PCR assay for *ctxA* (encoding subunit A of CTX) and *wbe* (encoding serogroup O1 antigen) (Hoshino *et al.*, 1998). These tests confirmed that the majority of the *V. cholerae* isolates associated with cholera in north-eastern Thailand were toxigenic and belonged to serogroup O1. Six serologically confirmed O1 strains reacted to monovalent Inaba antiserum and amplified primers for *ompW* and *wbe* (Hoshino *et al.*, 1998), but not *ctxA*, indicating that they were *ctx*$^{-}$ *V. cholerae* O1. The *ctx*$^{-}$ *V. cholerae* O1 occurs in the aquatic environment and has been shown to arise following loss of the CTX prophage (Alam *et al.*, 2007; Alam *et al.*, 2010).

Treatment of cholera patients with appropriate oral or intravenous rehydration, and a 1–3 day course of effective antibiotics (Saha *et al.*, 2006) can reduce the severity of infection, hospitalization, and faecal–oral transmission of cholera bacteria. Tetracycline (TE) has long been the drug of choice for the treatment of cholera, except for young children and pregnant women (Greenough *et al.*, 1964; Lindenbaum *et al.*, 1967). Other effective drugs have included furazolidone, erythromycin (E), trimethoprim/sulfamethoxazole (SXT), and chloramphenicol (Greenough *et al.*, 1964). However, antibiotic therapy has faced challenges related to the rapid emergence and spread of *V. cholerae* strains resistant to multiple antimicrobial agents, such as TE, ampicillin (AMP), kanamycin, streptomycin, SXT, nalidixic acid, E and most recently to ciprofloxacin and norfloxacin (Mhalu *et al.*, 1979; Glass *et al.*, 1980; Jain *et al.*, 2011). In this study, *V. cholerae* O1 strains from north-eastern Thailand isolated between 2003 and 2010 showed temporal variation in their response to different antibiotics. Although all strains isolated in 2007, two strains isolated in 2010, and one strain isolated in 2011 were MDR, strains isolated before and after 2007 were sensitive to the antibiotics tested and their response to different antibiotics varied from being sensitive to all, resistant to one, either E or SXT, and a few to AMP. Supawat *et al.* (2009) showed that some *V. cholerae* O1 strains isolated in Thailand between 2000 and 2004 were resistant to TE and SXT. In this study, *V. cholerae* associated with cholera between 2003 and 2004 showed resistance only to E and a few were also resistant to AMP, but none to TE and SXT. However, *V. cholerae* O1 strains isolated during 2007 in north-east Thailand were all resistant to four antibiotics, E, TE, SXT and AMP, although the patterns changed in 2010–2011, as all except three strains were sensitive to TE, with the rest either sensitive to either all or resistant to one, two or three of the antibiotics.

MDR *V. cholerae* strains isolated from cholera patients in Vietnam between 2008 and 2010 were resistant to four antibiotics, namely E, AMP, TE and SXT (Tran *et al.*, 2012), as occurred in Khon Kaen, Thailand, in 2007. Although Thailand shares its border with Vietnam and despite the fact that the resistant *V. cholerae* strains occurring in the two countries showed similar resistance patterns, it is highly unlikely that the resistant strains emerged in one of the two countries and were transmitted to the other, since resistant strains were present in Thailand in 2007 but not in 2010 when resistant strains were reported in Vietnam (Tran *et al.*, 2012). Most of the *V. cholerae* O1 isolated in Thailand between 2003 and 2011 were resistant to SXT, a recommended first-line drug for children with acute diarrhoea. In Thailand, TE and norfloxacin were the most frequently used drugs for treatment of cholera (Supawat *et al.*, 2009), but the data presented in this study on the emergence of MDR *V. cholerae* in Thailand, together with the increasing incidence of cholera caused by MDR *V. cholerae* in Africa, Asia, and South America (Glass *et al.*, 1980; Goel *et al.* 2011; Ibarra & Alvarado, 2007; Mandomando *et al.*, 2007; Tran *et al.*, 2012) indicate that drugs should not be administered without having sensitivity patterns first determined, considering that drug response patterns change frequently and can vary spatio-temporally.

In recent years, *V. cholerae* O1 ET has shown a shift in *ctxB* from genotype 3 (found in ET strains of the seventh pandemic and the Latin American epidemic) to genotype 1 (found in strains of CL biotype worldwide and the US Gulf Coast ET strains). Atypical ET possessing CL biotype CTX was first reported in Asia at the start of this century (Nair *et al.*, 2006; Raychoudhuri *et al.*, 2008; Morita *et al.*, 2010) and subsequently in Africa (Ansaruzzaman *et al.*, 2007) and Central America (Alam *et al.*, 2010), including Haiti (Chin *et al.*, 2011; Hasan *et al.*, 2012). Several genotypic variants of the Asian atypical (altered) ET were identified in Africa (Ansaruzzaman *et al.*, 2007; Choi *et al.*, 2010) and Central America (Alam *et al.*, 2010). In this study, *V. cholerae* O1 strains associated with cholera in Khon Kaen, Thailand, were phenotypically and genetically confirmed to be ET, but possessing CTX of the CL biotype, as reported previously in Thailand (Okada *et al.*, 2010; Na-Ubol *et al.* 2011). The data also show that, unlike the altered ET strains reported from Bangladesh (Nair *et al.*, 2006) and India (Raychoudhuri *et al.*, 2008), *V. cholerae* O1 ET strains isolated between 2003 and 2004, and one strain isolated in 2007 from a cholera outbreak in Khon Kaen, Thailand, did not carry *rstC*, a repressor gene sequence of the RS1 genetic element (Safa *et al.*, 2010) or the TLC gene cluster, which encodes a filamentous phage replicase and is proposed to be a satellite phage. Besides, all of these strains contained the repressor *rstR* gene (allele) of CL biotype, and thus appeared indistinguishable from the Mozambique variant of ET

carrying CL CTX prophage (Ansaruzzaman *et al.*, 2004; Faruque *et al.*, 2007). Interestingly, a sharp genetic transition of *V. cholerae* from the ET Mozambique variant to the Asian altered ET (possessing *ctxB* of the CL biotype) was observed in north-east Thailand in 2007, with all of the tested *V. cholerae* strains acquiring RS1 and TLC and switching the repressor *rstR* allele from $rstR^{CL}$ (CL) to $rstR^{ET}$ (ET), as in the altered ET reported from Bangladesh (Nair *et al.*, 2006) and India (Raychoudhuri *et al.*, 2008). Our data on the temporal absence of virulence and related genetic elements and their acquisitions clearly indicate horizontal gene transfer, which has been proposed to provide *V. cholerae* strains with improved evolutionary fitness (Faruque *et al.*, 2007).

A few *V. cholerae* O1 ET carrying a tandem repeat of the CL CTX prophage on the small chromosome, indistinguishable from the Mozambique ET variant, were isolated in Vietnam between 1995 and 2004 (Nguyen *et al.*, 2009). In a subsequent study, Tran *et al.* (2012) showed that *V. cholerae* O1 ET involved in recurrent cholera in Vietnam (2007–2010) was an ET variant carrying $ctxB^{CL}$ and $rstR^{ET}$ genes, as observed for ET strains involved in recurrent cholera in Khon Kaen, Thailand. Although the source of *V. cholerae* O1 ET analogous to the Mozambique variant of ET causing outbreaks in Thailand between 2003 and 2007 is not known, the Vietnamese ET strains carrying a tandem repeat of the CL CTX prophage and not involved in any major cholera outbreak were proposed to have been recently introduced into Vietnam (Nguyen *et al.*, 2009). Our observations of temporal fluctuation in resistance to antimicrobial agents and a genetic transition from Mozambique variant type ET to Asian altered ET suggest that *V. cholerae* has a natural reservoir (niche), which has allowed the bacterium to evolve locally in Khon Kaen, although the appearance of the *V. cholerae* O1 altered ET carrying $ctxB^{CL}$ and $rstR^{ET}$ alleles has been hypothesized to be a recent event in Thailand (Okada *et al.*, 2012).

Toxin co-regulated pilus (TCP), a type IV bundle-forming pilus of *V. cholerae*, is an essential colonization factor (Taylor *et al.*, 1987) that also serves as receptor for CTX-Φ (Waldor & Mekalanos, 1996). The genes encoding the biosynthesis of TCP pilus were shown to be encoded on a Vibrio pathogenicity island (VPI), a novel filamentous bacteriophage (Karaolis *et al.*, 1999). The TCP is a homopolymer of a 20.5 kDa major pilus protein, TcpA pilin (Taylor *et al.*, 1987) encoded by *tcpA*. The DNA sequence of *tcpA* differs slightly in the C-terminal domain for CL and ET biotype strains (Safa *et al.*, 2010). In the present study, the amino acid sequences of TcpA of *V. cholerae* O1 ET strains isolated in Thailand between 2003 and 2004 showed 100 % homology with the amino acid sequence of TcpA of ET reference strain N16961. However, the *tcpA* gene of *V. cholerae* O1 ET strains isolated in 2007 and thereafter had a mutation at amino acid position 64 (N→S). Although the change is subtle and it is not clear whether such genetic switching of the *tcpA* gene can provide *V. cholerae* O1 strains with increased environmental and/or epidemiological fitness (Faruque *et al.*, 2007), a similar change of amino acid at position 64 of TcpA was found in ctx^{-} *V. cholerae* O1 strains ZJ65 (2006) isolated in China (GenBank accession no. EU622532) and *V. cholerae* strain 2010EL1786 (GenBank accession no. CP003069) causing fatal epidemics in Haiti (Reimer *et al.*, 2011).

Historically, cholera has been endemic for centuries in the Ganges delta of the Bay of Bengal. Epidemic cholera in Africa and Latin America is suggested to have been introduced from cholera endemic countries of Asia (Blake, 1994). *V. cholerae* O1 ET populations involved in the 1991 cholera epidemic in Latin America were homogeneous initially (Blake, 1994), although divergent strains were detected later (Dalsgaard *et al.*, 1997; Beltrán *et al.*, 1999; Popovic *et al.*, 1993; Nusrin *et al.*, 2009). In Thailand, PFGE of *Not*I-digested genomic DNA revealed the majority of the *V. cholerae* O1 altered ET strains involved in recurrent cholera to be of a single clonal type, A, between 2003 and 2011, although more clonal types (B–D) mainly of surface water origin, were isolated in 2010 and thereafter, reflecting genetic diversity in the *V. cholerae* population. Evidence of an aquatic reservoir for *V. cholerae* is very extensive (Alam *et al.*, 2007), but the source and transmission of altered ET in Asia (Nair *et al.*, 2006), Africa (Ansaruzzaman *et al.*, 2004) and the Americas (Alam *et al.*, 2010; Chin *et al.*, 2011) remain enigmatic. Distinct signatures of clonally related altered ET strains in Thailand, Vietnam and Bangladesh, as revealed by PFGE (*Not*I)-based dendrograms, are presumed to be attributable to independent evolution of *V. cholerae* strains in different ecosystems. In any case, the findings of this study strongly suggest that cholera in north-east Thailand is endemic, caused by *V. cholerae* thriving locally and independent of cholera outbreaks in Vietnam and Bangladesh.

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