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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 possessedCTX 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 (NotI)-based dendrogram, suggesting that endemic cholera is caused by *V. cholerae* indigenous to Khon Kaen.

†These authors contributed equally to this work.

**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/sulfa-methoxazole (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 phase IV and Mukherjee ET phase 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 *ompW* PCR (Nandi et al., 2000). Serogroups were reconfirmed using multiplex PCR targeted at O1- (wboO1) and O139- (wboO139) specific O biosynthetic genes and the CTX gene (ctxA) (Hoshino et al., 1998). Biotype-specific characteristics were determined using PCR assays targeted to tcpA (CL and ET) (Rivera et al., 2001), rstR, encoding phase transcriptional regulator (Mwansa et al., 2007), presence of the repeat in toxin (rtxC) (Chow et al., 2001), rtIC, encoding an anti-repressor protein, and tle, 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<sup>CL</sup> and ctxB<sup>ET</sup>, 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'-ACTATCTTCAGATGAATGAG-3') and two allele-specific polymorphism detection primers, Rv-cl (5'-CTCTGTAACGATTCGATAG-3') and Rv-et (5'-CTCTGATACCTGACTTGAACA-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<sup>−1</sup> was applied at an included field angle of 120°. Genomic DNA of the test strains was

### Table 1. Primer primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Targeted gene</th>
<th>Annealing temp. (°C)</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ompW F</td>
<td>CAC CAA GAA GGT GAC TTT ATT GTG</td>
<td>ompW</td>
<td>64</td>
<td>304</td>
<td>Nandi et al. (2000)</td>
</tr>
<tr>
<td>ompW R</td>
<td>GGT TGG TCG AAT TAG CTG CAC C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rboO1 F</td>
<td>GGT CTA CTG AGC AGA TGG G</td>
<td>rboO1</td>
<td>55</td>
<td>192</td>
<td>Hoshino et al. (1998)</td>
</tr>
<tr>
<td>rboO1 R</td>
<td>GGT CAT CTG TAA GAA C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rboO139 F</td>
<td>AGC CTC TTT ATT ACG GGT GG</td>
<td>rboO139</td>
<td>55</td>
<td>449</td>
<td>Hoshino et al. (1998)</td>
</tr>
<tr>
<td>rboO139 R</td>
<td>GTC AAA CCC GAT CTT AAG GG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ctxA F</td>
<td>ACA GAG TGA GTA CTT TGA CC</td>
<td>ctxA</td>
<td>55</td>
<td>308</td>
<td>Hoshino et al. (1998)</td>
</tr>
<tr>
<td>ctxA R</td>
<td>ATC ACA TCA TTA TAT TGG GGA G</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tcpA ET R</td>
<td>CGA AAG CAC CTT CTT CCA CAC GTT G</td>
<td>tcpA ET</td>
<td>60</td>
<td>453</td>
<td>Rivera et al. (2001)</td>
</tr>
<tr>
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<td>CAC GAT AAG AAA ACC GGT CAA GAG</td>
<td>tcpA CL</td>
<td>60</td>
<td>620</td>
<td>Rivera et al. (2001)</td>
</tr>
<tr>
<td>tcpA R</td>
<td>CAC GAT AAG AAA ACC GGT CAA GAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAMA ETor F</td>
<td>ACT ATC TTC AGC ATA TCG ACG ACG</td>
<td>ctxB ET</td>
<td>55</td>
<td>186</td>
<td>Morita et al. (2008)</td>
</tr>
<tr>
<td>MAMA ETor R</td>
<td>CCT GGT ACT TCT ACT TGA AAC A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAMA class F</td>
<td>ACT ATC TTC AGC ATA TCG ACG ACG</td>
<td>ctxB CL</td>
<td>55</td>
<td>186</td>
<td>Morita et al. (2008)</td>
</tr>
<tr>
<td>MAMA class R</td>
<td>CCT GGT ACT TCT ACT TGA AAC A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rstR1 F</td>
<td>CTT TTC ATC TGC AAA GGC GCC TCA A</td>
<td>rstR CL</td>
<td>50</td>
<td>500</td>
<td>Mwansa et al. (2007)</td>
</tr>
<tr>
<td>rstR3A F</td>
<td>TCG AGT GTG AAT TCA TCA AGA GTG</td>
<td>rstR ET</td>
<td>50</td>
<td>500</td>
<td>Mwansa et al. (2007)</td>
</tr>
<tr>
<td>rstR2 R</td>
<td>GCA CCA TGA TTT AAG ATG CTC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rtxC F</td>
<td>CGA CGA AGA TCA TGG ACG AC</td>
<td>rtxC</td>
<td>55/56</td>
<td>265</td>
<td>Chow et al. (2001)</td>
</tr>
<tr>
<td>rtxC R</td>
<td>CAT GGT CTA TAT GTG GTG GC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rstC1</td>
<td>AAC AGC TAC GCG CTT ATT C</td>
<td>rstC</td>
<td>52.4</td>
<td>238</td>
<td>O’Shea et al. (2004)</td>
</tr>
<tr>
<td>rstC2</td>
<td>TGA GTG GCG GAT TTA GGC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tlc3</td>
<td>GGG AAT GTT GAG TTC TCA GTG</td>
<td>tlc</td>
<td>55.5</td>
<td>1548</td>
<td>O’Shea et al. (2004)</td>
</tr>
<tr>
<td>tlc4</td>
<td>GGT GCG AAG TAT TGG GTG</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>tcpA-F</td>
<td>ATG CAA TTA TTA AAA ACG CTT TTT AAG</td>
<td>tcpA</td>
<td>59</td>
<td>675</td>
<td>Kumar et al. (2010b)</td>
</tr>
<tr>
<td>tcpA-R</td>
<td>TTA GCT GTT ACC AAA TGC AAC AG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTX7</td>
<td>GGT TGG TCC TCA TCA TCA AAC CAC</td>
<td>ctxB</td>
<td>55</td>
<td>460</td>
<td>Olsvik et al. (1993)</td>
</tr>
<tr>
<td>CTX9B</td>
<td>GAT ACA CAT AAT AGA ATT AAG GAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

http://mm.sgmjournals.org
digested using *Ndo*I (Gibco-BRL), and *Salmonella enterica* serovar
Branderup 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
destaining. 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 recom-
ended 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 environ-
mental (*n*=3) isolates] included in this study produced colonies typical of *V. cholerae* on TTGA and gave
biochemical reactions characteristic for *V. cholerae*. 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
classical 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).

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**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

<table>
<thead>
<tr>
<th>Strains</th>
<th>Year of isolation</th>
<th>No. of isolates</th>
<th>Source*</th>
<th>Serotype</th>
<th><em>rfbO1</em></th>
<th>Phenotypic properties†</th>
<th>Genetic screening by PCR</th>
<th>Resistance pattern§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thai isolates</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>1</td>
<td>Clin Inaba</td>
<td>++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+ ET CL CL</td>
<td>E, AMP</td>
</tr>
<tr>
<td>2003</td>
<td>1</td>
<td>Env Inaba</td>
<td>++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+ ET CL CL</td>
<td>E, AMP</td>
</tr>
<tr>
<td>2004</td>
<td>1</td>
<td>Clin Inaba</td>
<td>++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+ ET CL CL</td>
<td>E</td>
</tr>
<tr>
<td>2004</td>
<td>1</td>
<td>Clin Inaba</td>
<td>++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+ ET CL CL</td>
<td>E</td>
</tr>
<tr>
<td>2004</td>
<td>1</td>
<td>Clin Inaba</td>
<td>++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+ ET CL CL</td>
<td>E</td>
</tr>
<tr>
<td>2007</td>
<td>1</td>
<td>Clin Ogawa</td>
<td>++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+ ET CL CL</td>
<td>E, TE, SXT, AMP</td>
</tr>
<tr>
<td>2010</td>
<td>1</td>
<td>Clin Ogawa</td>
<td>++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+ ET CL CL</td>
<td>E, TE, SXT, AMP</td>
</tr>
<tr>
<td>2010</td>
<td>1</td>
<td>Clin Ogawa</td>
<td>++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+ ET CL CL</td>
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<tr>
<td>2010</td>
<td>1</td>
<td>Clin Ogawa</td>
<td>++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+ ET CL CL</td>
<td>E</td>
</tr>
<tr>
<td>2010</td>
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<td>Clin Ogawa</td>
<td>++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+ ET CL CL</td>
<td>E</td>
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<tr>
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<td>2</td>
<td>Clin Ogawa</td>
<td>++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+ ET CL CL</td>
<td>E, SXT</td>
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<td>Clin Ogawa</td>
<td>++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+ ET CL CL</td>
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<td>Clin Ogawa</td>
<td>++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+ ET CL CL</td>
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<td>++</td>
<td>R</td>
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<td>2010</td>
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<td>Env Inaba</td>
<td>++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+ ET CL CL</td>
<td>SXT</td>
</tr>
<tr>
<td>2011</td>
<td>1</td>
<td>Clin Ogawa</td>
<td>++</td>
<td>R</td>
<td>R</td>
<td>R</td>
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<td>2011</td>
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<td>Clin Ogawa</td>
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| Reference strains | | | | | | | | |
| O395     | 1965   | –      | Clin Ogawa | ++ | R | R | + | ET | ET | E, AMP |
| N16961   | 1971   | –      | Clin Inaba | ++ | R | R | S | ET | ET | E, AMP |

*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 tcpAET, but not tcpACL. 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 ctxBCL, 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 *rstRCL*, but not the ET-biotype-specific repressor gene *rstRET*, 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 rtsC 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 rtsC and tlc and all carried the repressor *rstRET*, 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, rtsR and rtsC, 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/sulfamethoxazole, 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/sulfamethoxazole 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 NotI-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 (NotI) 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
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
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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 \textit{V. cholerae} from the ET Mozambique variant to the Asian altered ET (possessing \textit{ctxB} of the CL biotype) was observed in north-east Thailand in 2007, with all of the tested \textit{V. cholerae} strains acquiring RS1 and TLC and switching the repressor \textit{rstR} allele from \textit{rstR}^{CL} (CL) to \textit{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 \textit{V. cholerae} strains with improved evolutionary fitness (Faruque et al., 2007).

A few \textit{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 \textit{V. cholerae} O1 ET involved in recurrent cholera in Vietnam (2007–2010) was an ET variant carrying \textit{ctxB}^{CL} and \textit{rstR}^{ET} genes, as observed for ET strains involved in recurrent cholera in Khon Kaen, Thailand. Although the source of \textit{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 \textit{V. cholerae} has a natural reservoir (niche), which has allowed the bacterium to evolve locally in Khon Kaen, although the appearance of the \textit{V. cholerae} O1 altered ET carrying \textit{ctxB}^{CL} and \textit{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 \textit{V. cholerae}, is an essential colonization factor (Taylor et al., 1987) that also serves as receptor for CTX-\textit{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 (Karolis et al., 1999). The TCP is a homopolymer of a 20.5 kDa major pilus protein, TcpA pilin (Taylor et al., 1987) encoded by \textit{tcpA}. The DNA sequence of \textit{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 \textit{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 \textit{tcpA} gene of \textit{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 \textit{tcpA} gene can provide \textit{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 \textit{ctx}^+ \textit{V. cholerae} O1 strains ZJ65 (2006) isolated in China (GenBank accession no. EU622532) and \textit{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). \textit{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 NotI-digested genomic DNA revealed the majority of the \textit{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 \textit{V. cholerae} population. Evidence of an aquatic reservoir for \textit{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 (NotI)-based dendograms, are presumed to be attributable to independent evolution of \textit{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 \textit{V. cholerae} thriving locally and independent of cholera outbreaks in Vietnam and Bangladesh.

**ACKNOWLEDGEMENTS**

This study was partly supported by research grants from the Faculty of Medicine, Khon Kaen University and Department of Disease Control, Ministry of Public Health, Thailand National Institutes of Health Grant No. RO1AI039129, under collaborative agreements between the Johns Hopkins Bloomberg School of Public Health, the University of Maryland, College Park, and the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). ICDDR,B acknowledges its major donors: Australian Agency for International Development (AusAID), Government of the People’s Republic of Bangladesh, Canadian International Development Agency (CIDA), Swedish International Development Cooperation Agency (SIDA), and the Department for International Development, UK (DFID). C.C. thanks the staff at the Office of Communicable Disease Control, Region 6, Khon Kaen, for their help with specimen collection.

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