Multilocus variable-number tandem repeat analysis of Vibrio cholerae O1 El Tor strains harbouring classical toxin B

Seon Young Choi,1,2 Je Hee Lee,1,2 Yoon-Seong Jeon,1,2 Hye Ri Lee,1 Eun Jin Kim,1 M. Ansaruzzaman,3 Nurul A. Bhuiyan,3 Hubert P. Endtz,3 S. K. Niyogi,4 B. L. Sarkar,4 G. Balakrish Nair,4 Binh Minh Nguyen,5 Nguyen Tran Hien,5 Cecil Czerkinsky,1 John D. Clemens,1 Jongsik Chun1,2 and Dong Wook Kim1

1International Vaccine Institute, Seoul, Republic of Korea
2School of Biological Sciences, Seoul National University, Seoul, Republic of Korea
3International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), Dhaka, Bangladesh
4National Institute of Cholera and Enteric Diseases, Kolkata, India
5National Institute of Hygiene and Epidemiology, Hanoi, Vietnam

Atypical Vibrio cholerae O1 strains – hybrid strains (strains that cannot be classified either as El Tor or classical biotype) and altered strains (El Tor biotype strains that produce classical cholera toxin) – are currently prevalent in Asia and Africa. A total of 74 hybrid and altered strains that harboured classical cholera toxin were investigated by multilocus variable-number tandem repeat analysis (MLVA). The results showed that the hybrid/altered strains could be categorized into three groups and that they were distant from the El Tor strain responsible for the seventh cholera pandemic. Hybrid/altered strains with a tandem repeat of the classical CTX prophage on the small chromosome were divided into two MLVA groups (group I: Mozambique/Bangladesh group; group III: Vietnam group), and altered strains with the RS1–CTX prophage containing the El Tor type rstR and classical ctxB on the large chromosome were placed in two MLVA groups (group II: India/Bangladesh group; group III: India/Vietnam group).

INTRODUCTION

Cholera is a severe diarrhoeal disease caused by Vibrio cholerae (Kaper et al., 1995; Sack et al., 2004). Among the more than 200 serogroups of V. cholerae, only O1 and O139 cause epidemic cholera. The O1 serogroup has been divided into three serotypes, Ogawa, Inaba and Hikojima (Kaper et al., 1995), and into two biotypes, classical and El Tor. Classical biotype strains are considered to be responsible for the first six cholera pandemics since the early 19th century, whilst El Tor biotype strains are responsible for the current (seventh) cholera pandemic, which began in 1961 (Kaper et al., 1995; Sack et al., 2004).

Since the early 1990s, new variants of the V. cholerae O1 serogroup – hybrid and altered strains – have emerged and have entirely replaced prototype El Tor strains in Asian countries and Mozambique (Lee et al., 2006; Nair et al., 2002, 2006; Nguyen et al., 2009). Hybrid strains were initially defined as atypical strains that could not be biotyped as classical or El Tor and alternatively named the Matlab variants (Nair et al., 2002). Hybrid strains are reported to harbour the El Tor type cholera toxin (CT) B subunit gene (ctxB) and/or classical ctxB, whilst altered strains contain classical ctxB (Safa et al., 2006). The El Tor biotype strains that harbour classical ctxB have been designated altered strains (Nair et al., 2006). Recently, an alternative nomenclature for the hybrid and altered strains has been suggested, with ‘El Tor variants’ as well as a new biotyping scheme for V. cholerae O1 (Raychoudhuri et al., 2008; Safa et al., 2010). Two different genetic structures of the CTX prophage and RS1 element on each chromosome have been reported among the hybrid and altered strains (Lee et al., 2006, 2009; Nguyen et al., 2009). As there are different CTX prophage arrays on different chromosomes among these, we postulated that these strains could have differences in their genomes. However, when we applied multilocus sequence typing analysis to the altered strains, most contained the same sequence type as the prototype El Tor strain, N16961 (Lee et al., 2006). A number of
multilocus variable-number tandem repeat analysis (MLVA) studies of *V. cholerae* strains collected mainly in Bangladesh and India have been reported (Danin-Poleg et al., 2007; Ghosh et al., 2008; Stine et al., 2008). We therefore employed MLVA to find relevant genetic differences among the hybrid/altered strains, which were collected mainly in India and Bangladesh. We found that hybrid/altered strains could be categorized into three groups. Strains collected in Mozambique and some strains from Bangladesh, which had a tandem repeat of the CTX prophage on the small chromosome, could be grouped together, whilst all analysed Asian strains containing the RS1–CTX prophage array on the large chromosome were subdivided into two groups.

**METHODS**

**V. cholerae strains.** A total of 77 *V. cholerae* O1 isolates, including strain N16961 (biotype El Tor), O395 and 569B (biotype classical) for reference, were analysed in this study. B33, which has a tandem repeat of the classical CTX prophage on the small chromosome, and four isolates collected together with B33 in Mozambique in 2004 were included (Faruque et al., 2007; Lee et al., 2006). Nineteen *V. cholerae* isolates collected from northern Vietnam (ten isolates collected from 1995 to 2004 and nine collected during cholera outbreaks in 2007–2008), 16 isolates from the International Centre for Diarrhoeal Disease Research, Bangladesh, and 34 randomly selected isolates from a collection of 424 clinical isolates from Kolkata, India (collected from 2003 to 2007) were analysed (Lee et al., 2006; Nguyen et al., 2009; Roychowdhury et al., 2008).

The CTX prophage and RS1 array and the ctxB sequence of the *V. cholerae* strains were determined as described previously (Lee et al., 2009; Nguyen et al., 2009).

**Genetic analysis.** Genomic DNA was prepared from agar-grown cultures using a Prepman Ultra kit (Applied Biosystems). Five loci for MLVA were amplified using primers and PCR conditions as described in previous studies (Ghosh et al., 2008; Stine et al., 2008) and in Table 1. The purified PCR products were sequenced in both directions using a Big Dye Cycle Sequencing kit (Applied Biosystems) and sequencing was performed on an ABI 3770 automatic sequencer according to the manufacturer’s instructions. Sequence data for each isolate were added to a group of known sequences, which were aligned simultaneously and edited through PHYLIP (Leon et al., 2005). MLVA types were assigned by combining numbers of repeat units of each locus in the order described above. Minimum spanning trees were generated using BioNumerics (Applied Maths) based on the categorical coefficient.

**RESULTS AND DISCUSSION**

**V. cholerae strains and their CTX prophage**

Two strains, MJ1236 and MG116926, are known as hybrid strains or Matlab variants I and III, respectively, and contain a tandem repeat of the classical CTX prophage on the small chromosome (Lee et al., 2009; Safa et al., 2006). All other strains, except for N16961 (El Tor biotype), O395 and 569B (classical biotype), were altered strains containing either a tandem repeat of the classical CTX prophage on the small chromosome (ten isolates collected in Vietnam from 1995 to 2004, E1781 collected in Bangladesh and five Mozambican isolates) or harbouring an El Tor type CTX prophage with classical ctxB on the large chromosome (Nguyen et al., 2009). Detailed information on the isolates, including the year and country of isolation and the CTX prophage and RS1 element array on each chromosome, is listed in Table 2.

**MLVA analysis of V. cholerae strains**

The MLVA scheme that was applied previously to *V. cholerae* strains from Bangladesh and India was used in this study (Ghosh et al., 2008; Stine et al., 2008). Instead of using arbitrary allele type numbers, we used the number of repeats of each locus directly in this study. In the previous studies, a new allele type number was assigned to represent the number of repeats of each locus; however, the number of repeats of each locus is shown directly in this study. The MLVA profile was assigned by combining the number of repeats of five loci. Compared with the three loci on the large chromosome (VC0147, VC0436-7 and VC1650), on which five, four and five different allele types were identified, respectively, two loci on the small chromosome

### Table 1. MLVA loci characteristics and the number of alleles identified in this study

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Locus</th>
<th>Repeat unit</th>
<th>Primers (5’→3’)</th>
<th>No. of alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VC0147 [cell division protein (<em>ftsY</em>)]</td>
<td>AACAGA</td>
<td>CCAAACCACGTGCAACGGATA*</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>VC0436-7 [non-coding (intergenic)]</td>
<td>GACCTCA</td>
<td>CGTTGCTGACTAAGTTCACGC</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>VC1650 [collagenase]</td>
<td>GATATCCA</td>
<td>CTACCAAGGGCGGTTAAGCTG</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>VCA0171 [hypothetical protein]</td>
<td>TGCTGT</td>
<td>GCATCTACACAGGGTTTGG</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>VCA0283 [hypothetical protein]</td>
<td>ACCAGA</td>
<td>CTTATCGGCCCAAAACAGACA*</td>
<td>20</td>
</tr>
</tbody>
</table>

*Primers designed in this study based on previous reports. Other primers have been described previously (Ghosh et al., 2008; Stine et al., 2008).*
Table 2. V. cholerae strains and isolates analysed in this study

The country and year in which each isolate was collected, CTX prophage and RS1 element array on each chromosome, and MLVA profile are indicated. Allele numbers represent the total number of repeats of each locus. CTXET, CTX prophage containing El Tor rstR and El Tor ctxB; CTXcl, CTX prophage containing classical rstR and classical ctxB; CTXec, CTX prophage containing El Tor rstR and classical ctxB; TLC, toxin-linked cryptic element.

<table>
<thead>
<tr>
<th>Original ID</th>
<th>Country, year</th>
<th>Chromosome 1</th>
<th>Chromosome 2</th>
<th>VC0147</th>
<th>VC0436-7</th>
<th>VC1650</th>
<th>VCA0171</th>
<th>VCA0283</th>
<th>MLVA group</th>
</tr>
</thead>
<tbody>
<tr>
<td>O395</td>
<td>Reference strain, classical</td>
<td>TLC-truncated CTXcl–CTXcl</td>
<td>CTXcl</td>
<td>7</td>
<td>4</td>
<td>3</td>
<td>24</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>569B</td>
<td>Reference strain, classical</td>
<td>TLC-truncated CTXcl–CTXcl</td>
<td>CTXcl</td>
<td>10</td>
<td>4</td>
<td>3</td>
<td>15</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>N16961</td>
<td>Reference strain, El Tor</td>
<td>TLC–CTXET–RS1</td>
<td>–</td>
<td>9</td>
<td>7</td>
<td>7</td>
<td>23</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

B-33 Mozambique, 2004 – CTXcl–CTXcl 8 7 8 11 20 I
B-81 Mozambique, 2004 – CTXcl–CTXcl 8 7 8 11 20 I
042-3 Mozambique, 2004 – CTXcl–CTXcl 8 7 8 11 22 I
127-1 Mozambique, 2004 – CTXcl–CTXcl 7 7 8 11 20 I
308-1 Mozambique, 2004 – CTXcl–CTXcl 8 7 8 11 21 I
07.95/Vc.P Vietnam, 1995 CTXcl CTXcl–CTXcl 10 6 8 16 26 III
32.02/Vc.P Vietnam, 2002 CTXcl CTXcl–CTXcl 10 6 8 16 26 III
272.03/Vc.P Vietnam, 2003 – CTXcl–CTXcl 10 6 8 17 28 III
43.04/Vc.P Vietnam, 2004 CTXcl CTXcl–CTXcl 10 6 8 16 29 III
55.04/Vc.P Vietnam, 2004 CTXcl CTXcl–CTXcl 10 6 8 16 29 III
62.04/Vc.P Vietnam, 2004 CTXcl CTXcl–CTXcl 10 6 8 16 29 III
71.04/Vc.P Vietnam, 2004 CTXcl CTXcl–CTXcl 10 6 8 16 29 III
73.04/Vc.P Vietnam, 2004 CTXcl CTXcl–CTXcl 10 6 8 16 29 III
84.04/Vc.P Vietnam, 2004 CTXcl CTXcl–CTXcl 10 6 8 16 26 III
01.07/Vc.P Vietnam, 2007 TLC–RS1–CTXcl–CTXcl 10 6 7 16 16 16 III
34.07/Vc.P Vietnam, 2007 TLC–RS1–CTXcl–CTXcl 10 6 7 15 17 17 III
69.07/Vc.C* Vietnam, 2007 TLC–RS1–CTXcl–CTXcl 10 6 7 15 17 17 III
629.07/Vc.W* Vietnam, 2007 TLC–RS1–CTXcl–CTXcl 10 6 7 16 16 16 III
226.08/Vc.P Vietnam, 2008 TLC–RS1–CTXcl–CTXcl 10 6 7 16 16 16 III
491.08/Vc.V* Vietnam, 2008 TLC–RS1–CTXcl–CTXcl 10 6 7 17 19 19 III
1056.08/Vc.C* Vietnam, 2008 TLC–RS1–CTXcl–CTXcl 10 6 7 17 19 19 III
2061.08/Vc.P* Vietnam, 2008 TLC–RS1–CTXcl–CTXcl 10 6 7 16 16 16 III
VC073 Bangladesh, 1994 TLC–RS1–CTXcl–CTXcl 10 6 7 16 16 16 III
MQ4 Bangladesh, 2001 TLC–RS1–CTXcl–CTXcl 10 6 7 16 17 17 II
MQ1194 Bangladesh, 2001 TLC–RS1–CTXcl–CTXcl 10 6 7 10 10 10 II
MQ1273 Bangladesh, 2001 TLC–RS1–CTXcl–CTXcl 10 6 7 10 10 10 II
MQ1356 Bangladesh, 2001 TLC–RS1–CTXcl–CTXcl 10 6 7 21 12 II
MQ1379 Bangladesh, 2001 TLC–RS1–CTXcl–CTXcl 10 6 7 10 10 10 II
MQ1795 Bangladesh, 2001 TLC–RS1–CTXcl–CTXcl 10 6 7 16 16 16 II
MQ2200 Bangladesh, 2001 TLC–RS1–CTXcl–CTXcl 10 6 7 15 15 15 II
CRS101 Bangladesh, 2002 TLC–RS1–CTXcl–CTXcl 10 6 7 16 16 16 II
MJ1236 Bangladesh, 1994 TLC–RS1–CTXcl–CTXcl 8 7 8 12 19 19 I
MG116926 Bangladesh, 1991 TLC–RS1–CTXcl–CTXcl 8 7 8 14 23 23 I
E1781* Bangladesh, 2000 TLC–RS1–CTXcl–CTXcl 8 7 8 9 24 24 I
E1978* Bangladesh, 2000 TLC–RS1–CTXcl–CTXcl 9 7 6 16 8 8 II
E1797* Bangladesh, 2001 TLC–RS1–CTXcl–CTXcl 9 7 6 16 8 8 II
EC1293* Bangladesh, 1999 TLC–RS1–CTXcl–CTXcl 9 7 6 15 15 15 II
AR32732 Bangladesh, 2004 TLC–RS1–CTXcl–CTXcl 9 7 6 22 22 22 II
7 India, 2004 TLC–RS1–CTXcl–CTXcl 9 7 6 19 19 19 II
26 India, 2005 TLC–RS1–CTXcl–CTXcl 9 7 6 19 19 19 II
87 India, 2004 TLC–RS1–CTXcl–CTXcl 11 7 7 14 14 14 III
104 India, 2006 TLC–RS1–CTXcl–CTXcl 11 7 7 14 14 14 III
(VCA0171 and VCA0283) were shown to be more variable, as the allele numbers of these loci were 15 and 20, respectively (Table 1).

Overall, the *V. cholerae* O1 strains analysed in this study could largely be divided into three MLVA profile groups: group I, strains containing MLVA profile 8,7,8,X,X; group II, strains with the MLVA profile 9,7,6,X,X, or 9,3,6,X,X; and group III, strains with the MLVA profile 10,6,7,X,X (Fig. 1 and Table 2). Based on the finding that the last two loci on the small chromosome were not the determinant factor of this grouping, we denoted these two loci as X.

The Mozambican isolates and Bangladeshi strains MJ1236, MG116926 and E1781 had a tandem repeat of the CTX prophage on the small chromosome and had the MLVA profile 8,7,8,X,X (one variant with the profile 7,7,8,X,X was found in Mozambican strain 127-1). This implied that these strains were more closely related than other altered strains with respect not only to their CTX prophage array but also to their genomic variations. The other 13 Bangladeshi isolates contained the RS1–CTX prophage (El Tor *rstR* and classical *ctxB*) array on the large chromosome and belonged to MLVA profile group II with a number of variations (Table 2).

The Indian isolates belonged to one of two MLVA profiles: group II (9,3,6,X,X or 9,7,6,X,X) and group III (10,6,7,X,X). All had an RS1–CTX prophage array on the large chromosome. The CTX prophage on the large chromosome contained the El Tor type *rstR* and the classical type *ctxB*. A number of Indian isolates had an additional amino acid change on the classical *ctxB* background (CT genotype 6) (Goel et al., 2008). This variant *ctxB* was found mainly in group II (9,3,6,X,X); however, a few isolates were also identified in group III (11,6,7,X,X). This suggested that the changes in *ctxB* were independent events from changes on the genome. None of the Indian isolates fitted into group I; however, we cannot exclude the possibility of the presence of strains belonging to group I in India. As the Indian isolates analysed in this study were collected recently (2004–2007) and only from Kolkata, more diverse groups might be found among

### Table 2. cont.

<table>
<thead>
<tr>
<th>Original ID</th>
<th>Country, year</th>
<th>Chromosome 1</th>
<th>Chromosome 2</th>
<th>VC0147</th>
<th>VC0436-7</th>
<th>VC1650</th>
<th>VCA0171</th>
<th>VCA0283</th>
<th>MLVA group</th>
</tr>
</thead>
<tbody>
<tr>
<td>178</td>
<td>India, 2006</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>9</td>
<td>3</td>
<td>6</td>
<td>19</td>
<td>17</td>
<td>II</td>
</tr>
<tr>
<td>200</td>
<td>India, 2006</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>9</td>
<td>3</td>
<td>6</td>
<td>22</td>
<td>19</td>
<td>II</td>
</tr>
<tr>
<td>214</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>9</td>
<td>3</td>
<td>6</td>
<td>19</td>
<td>16</td>
<td>II</td>
</tr>
<tr>
<td>236</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>9</td>
<td>3</td>
<td>6</td>
<td>17</td>
<td>16</td>
<td>II</td>
</tr>
<tr>
<td>301</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>10</td>
<td>6</td>
<td>6</td>
<td>14</td>
<td>18</td>
<td>III</td>
</tr>
<tr>
<td>302</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>10</td>
<td>6</td>
<td>6</td>
<td>14</td>
<td>18</td>
<td>III</td>
</tr>
<tr>
<td>303</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>9</td>
<td>3</td>
<td>6</td>
<td>19</td>
<td>16</td>
<td>II</td>
</tr>
<tr>
<td>304</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>9</td>
<td>3</td>
<td>6</td>
<td>19</td>
<td>16</td>
<td>II</td>
</tr>
<tr>
<td>305</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>10</td>
<td>6</td>
<td>7</td>
<td>14</td>
<td>14</td>
<td>III</td>
</tr>
<tr>
<td>315</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>10</td>
<td>6</td>
<td>7</td>
<td>14</td>
<td>14</td>
<td>III</td>
</tr>
<tr>
<td>316</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>10</td>
<td>6</td>
<td>7</td>
<td>15</td>
<td>15</td>
<td>III</td>
</tr>
<tr>
<td>317</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>10</td>
<td>6</td>
<td>7</td>
<td>16</td>
<td>14</td>
<td>III</td>
</tr>
<tr>
<td>324</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>10</td>
<td>7</td>
<td>7</td>
<td>14</td>
<td>19</td>
<td>III</td>
</tr>
<tr>
<td>340</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>9</td>
<td>3</td>
<td>6</td>
<td>20</td>
<td>18</td>
<td>II</td>
</tr>
<tr>
<td>345</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>10</td>
<td>6</td>
<td>7</td>
<td>15</td>
<td>15</td>
<td>III</td>
</tr>
<tr>
<td>350</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>10</td>
<td>6</td>
<td>7</td>
<td>15</td>
<td>19</td>
<td>III</td>
</tr>
<tr>
<td>360</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>10</td>
<td>6</td>
<td>7</td>
<td>15</td>
<td>15</td>
<td>III</td>
</tr>
<tr>
<td>365</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>10</td>
<td>6</td>
<td>7</td>
<td>14</td>
<td>20</td>
<td>III</td>
</tr>
<tr>
<td>370</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>10</td>
<td>6</td>
<td>7</td>
<td>15</td>
<td>15</td>
<td>III</td>
</tr>
<tr>
<td>380</td>
<td>India, 2006</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>10</td>
<td>6</td>
<td>7</td>
<td>14</td>
<td>21</td>
<td>III</td>
</tr>
<tr>
<td>386</td>
<td>India, 2006</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>10</td>
<td>6</td>
<td>7</td>
<td>14</td>
<td>21</td>
<td>III</td>
</tr>
<tr>
<td>406</td>
<td>India, 2006</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>11</td>
<td>6</td>
<td>7</td>
<td>14</td>
<td>18</td>
<td>III</td>
</tr>
<tr>
<td>407</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>9</td>
<td>3</td>
<td>6</td>
<td>17</td>
<td>16</td>
<td>II</td>
</tr>
<tr>
<td>410</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>9</td>
<td>3</td>
<td>6</td>
<td>19</td>
<td>17</td>
<td>II</td>
</tr>
<tr>
<td>411</td>
<td>India, 2005</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>11</td>
<td>6</td>
<td>7</td>
<td>14</td>
<td>18</td>
<td>III</td>
</tr>
<tr>
<td>412</td>
<td>India, 2006</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>11</td>
<td>6</td>
<td>7</td>
<td>14</td>
<td>18</td>
<td>III</td>
</tr>
<tr>
<td>419</td>
<td>India, 2006</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>9</td>
<td>3</td>
<td>6</td>
<td>20</td>
<td>25</td>
<td>II</td>
</tr>
<tr>
<td>420</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>11</td>
<td>6</td>
<td>7</td>
<td>14</td>
<td>18</td>
<td>III</td>
</tr>
<tr>
<td>421</td>
<td>India, 2006</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>11</td>
<td>6</td>
<td>7</td>
<td>14</td>
<td>18</td>
<td>III</td>
</tr>
<tr>
<td>423</td>
<td>India, 2007</td>
<td>TLC–RS1–CTX</td>
<td>–</td>
<td>11</td>
<td>6</td>
<td>7</td>
<td>14</td>
<td>18</td>
<td>III</td>
</tr>
</tbody>
</table>

*Environmental isolates. All others are clinical isolates.*
isolates collected earlier and from other regions. Chatterjee et al. (2009) suggested that some of the Indian isolates collected in 1992 could be considered the progenitors of the Mozambican strains based on CTX prophage array and ribotyping results.

All the Vietnamese isolates belonged to group III (10,6,7,X,X), but we found considerable changes in the CTX prophage array with accompanying changes in the MLVA profile in the span of just a few years (Fig. 1). Among the isolates collected from 1995 to 2004, isolates having a tandem repeat of the classical CTX prophage on the small chromosome had an MLVA profile 10,6,8,17,28, whilst isolates with an additional classical CTX prophage on the large chromosome had MLVA profile 10,6,8,16,26 or 10,6,8,16,29 (Table 2). The isolates collected during cholera outbreaks in 2007 and 2008 had an RS1–CTX prophage array with El Tor rstR and classical ctxB on the large chromosome, which was a distinguishable characteristic compared with earlier isolates (Nguyen et al., 2009). The MLVA profile of these isolates was 10,6,7,X,X, which was also different from the earlier isolates. Although strains with the 10,6,7,X,X profile and RS1–CTX prophage array on the large chromosome were also found in India, strains with an MLVA profile of 10,6,8,X,X and a tandem repeat of the classical CTX prophage on the small chromosome were not found in India or in Bangladesh (Table 2).

In previous reports, O1 strains belonging to group II (9,3,6,X,X profile) and O139 strains belonging to group I (8,7,8,22,23, 8,7,8,21,23 and 8,7,8,21,16) were identified in Bangladesh (Stine et al., 2008). The Bangladeshi V. cholerae O1 serogroup strains analysed in our study belonged to group I or group II. These O139 strains and O1 strains

![Fig. 1. Minimum spanning tree (based on the categorical coefficient and generated by BioNumerics software; Applied Maths) of V. cholerae strains based on MLVA. Countries in which the isolates were collected are indicated by different types of circle. Strains belonging to group I and Vietnamese isolates collected between 1995 and 2004 in group III contained a tandem repeat of the classical CTX prophage on the small chromosome (simple diagrams of the CTX prophage and RS1 element array on both chromosomes are indicated). All others contained the RS1–CTX prophage array with El Tor rstR (ET) and classical ctxB (cla) on the large chromosome. Isolates that had an additional amino acid change on the classical ctxB background are marked with an asterisk. The MLVA profiles of reference strains N16961 (9,7,7,23,14), O395 (7,4,3,24,14) and 569B (10,4,3,15,39) are distant from those of the hybrid/altered strains and are not shown on the tree. TLC, Toxin-linked cryptic element.]
belonging to group I should be analysed further to identify the origin of altered strains and O139 strains. Group II (9,3,6,X,X) and group III (11,7,7,14,X) strains were found throughout India as described previously (Ghosh et al., 2008).

Hybrid and altered strains have been isolated since the early 1990s, as has the O139 serogroup (Chatterjee et al., 2009). An extensive study analysing O1 El Tor and O139 serogroup strains collected during that period is necessary to investigate the genetic origin of the O139 serogroup and O1 hybrid/altered strains of V. cholerae. The results in this report suggest that a group of El Tor strains (group I strains and earlier Vietnamese strains) obtained the classical CTX prophage, which integrated into the small chromosome. The El Tor type ctxB on the El Tor CTX prophage of other groups of strains (groups II and III) was replaced by the classical ctxB. We should not rule out the possibility that the group II and III strains could have merged as they have the same RS1–CTX prophage array.

A possible generation mechanism of the Mozambican strain from El Tor strains has been suggested (Faruque et al., 2007); however, further genetic analyses including whole-genome sequencing of diverse strains, prototype El Tor strains and hybrid/altered strains are required to elucidate the origin of hybrid/altered strains. Among the strains analysed in this study, we found that B33 and MJ1236 produced significantly more CT than other strains under in vitro AKI conditions (Iwanga et al., 1986). Detailed analyses of the amount of toxin they produce, the possible mechanism of this difference and a link between the degree of virulence of these strains and their toxin production abilities is under way.

In conclusion, three MLVA profile groups were identified among the hybrid and altered strains of V. cholerae O1 serogroup analysed in this study. We found that strains with a tandem repeat of the classical CTX prophage on the small chromosome could be categorized into two MLVA profile groups (group I with MLVA profile 8,7,8,X,X, collected in Mozambique and Bangladesh, and group III with MLVA profile 10,6,8,X,X collected in Vietnam). Strains with the RS1–CTX prophage (El Tor type rstR and classical ctxB) array on the large chromosome could be classified into two groups: group II strains with an MLVA profile of 9,3,6,X,X/9,7,6,X,X were found in India and Bangladesh, and group III strains containing the MLVA profile 10,6,7,X,X were found in India and Vietnam.

ACKNOWLEDGEMENTS

This work was supported by the Cholera Vaccine Initiative, funded by the Bill and Melinda Gates Foundation. The International Vaccine Institute is supported by the Governments of Korea, Sweden and Kuwait. H. R. L., H. H. L. and D. W. K. were supported by grant RTI05-01-01 from the Regional Technology Innovation Program of the Ministry of Knowledge and Economy (MKE), Republic of Korea.

REFERENCES


