Emergence and genetic diversity of El Tor *Vibrio cholerae* O1 that possess classical biotype *ctxB* among travel-associated cases of cholera in Japan

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INTRODUCTION

*Vibrio cholerae* O1 are classified into two biotypes, classical and El Tor, each encoding a biotype-specific cholera toxin. However, El Tor strains have recently emerged with a classical cholera-toxin genotype (El Tor variant). We characterized El Tor strains of *V. cholerae* O1 from travel-associated cases of cholera in Japan isolated from 1991 to 2006 by cholera toxin B subunit gene (*ctxB*) typing and by molecular epidemiological methods. *ctxB* in the biotype El Tor shifted from the El Tor-specific type to the classical-specific type around 1993, and this type fully dominated the later half of the 1990s. Based on the results of PFGE and multilocus variable-number tandem repeat analysis, strains of the classical biotype remained diverse from those of El Tor biotype. The El Tor biotype strains formed multiple minor clusters and intermingled with each other irrespective of their origins and toxin types. El Tor variant strains are widespread in Asian countries and show significant genetic diversity, indicating that their spread is a result of monoclonal expansion rather than spread from a single clone.

Abbreviations: MAMA-PCR, mismatch amplification mutation assay PCR; MLVA, multilocus variable-number tandem repeat analysis.
to replace the typical El Tor strains in Asian endemic areas. El Tor variant strains showed a variety of genotypes for molecular epidemiology, which suggests that they originated from multiple geographical areas.

METHODS

Bacterial strains. We collected 67 clinical strains of V. cholerae O1 from travel-associated cholera cases in Japan between 1991 and 2006. Sixty-four were from patients with a history of travel to Asian countries, two were from patients who had travelled to Latin America (Bolivia and Peru) in 1992 and one was from Africa (Madagascar) in 2000. The fully genome sequenced El Tor strain N16961 and ten typical classical strains were used to replace the typical El Tor strains in Asian endemic areas. Sixty-four were from patients with a history of travel to Asian countries, two were from patients who had travelled to Latin America (Bolivia and Peru) in 1992 and one was from Africa (Madagascar) in 2000. The fully genome sequenced El Tor strain N16961 and ten typical classical strains were also related to molecular epidemiological studies for use as reference strains. The biotypes of the strains were confirmed by testing chicken erythrocyte agglutination, and sensitivity to polymyxin B, Mukerjee classical plaque IV and Bus and Mukerjee El Tor plaque V.

ctxB typing by MAMA-PCR. MAMA-PCR was employed to detect sequence polymorphism between classical and El Tor ctxB genes at nucleotide position 203 for toxin typing (Morita et al., 2008). Brieﬂy, ampliﬁcation of the ctxB gene was accomplished using primer 5’-ACTATCCTTGAGCATACATGGCAATG-3’ (Fw-con), with 5’-CCTGG-TTCTCTAATGGAACG-3’ (Rv-cla, specific for the classical ctxB) or 5’-CCTGGTACTCTTACTTCTGAAAACA-3’ (Rv-elt, speciﬁc for the El Tor ctxB), in PCR mixture (10 μl) containing 1 μl 10 × Ex Taq buffer (Takara Bio), 100 μM dNTPs and 0.5 μl overnight strain culture with 0.25 U Ex Taq (Takara Bio). 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 55°C for 10 s and extension at 72°C for 30 s; and ﬁnal extension at 72°C for 2 min. The ampliﬁed fragments were detected after 3% agarose gel electrophoresis and staining with ethidium bromide (0.1 μg ml−1).

PFGE. PFGE was performed as previously described using the Salmonella enterica serovar Braenderup H9812 as a standard strain (Cooper et al., 2006). The DNA in agarose plugs was digested with NotI (Promega). Digested DNA was separated through a 1% SeaKem Gold agarose gel (Cambrex Bio Science) in 0.5 × TBE buffer [50 mM Tris, 45 mM boric acid, 0.5 mM EDTA (pH 8.4)] at 14°C in a CHEF DR-III (Bio-Rad Laboratory) under the following electrophoresis conditions: switch time of 2–10 s for 13 h and 20–25 s for 6 h, 6 V cm−1, at an angle of 120°. The resulting proﬁles were scanned and saved in TIFF format to be analysed using BioNumerics software (Applied Math). Similarity was determined using the Dice coeﬃcient, and clustering was based on UPGMA with a band position tolerance of 1.2%.

Multilocus variable-number tandem repeat analysis (MLVA).

MLVA of V. cholerae was performed using the loci described by Danin-Poleg et al. (2007) with modiﬁcations. Seven loci were selected and applied in this study. The ﬁve loci (locus names 1, 2, 3, 5 and 6) were on the large chromosome and the two loci (locus names 7 and 8) were on the small chromosome. The details of the loci and primers are listed in Table 2. V. cholerae O1 strains were cultivated on nutrient agar with 1% sodium chloride. DNA was extracted from fresh colonies using QuickGene-810 with the QuickGene DNA tissue kit S (Fujifilm). The seven selected variable number of tandem repeats loci were ampliﬁed in a single multiplex PCR. The PCR mixture contained 0.2 μM each primer, 1 μl DNA template and 1 × multiplex PCR mixture (Qiagen). The PCR conditions were as follows: initial denaturation at 95°C for 15 min; 35 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 90 s and extension at 72°C for 60 s; and ﬁnal extension at 72°C for 10 min.

RESULTS AND DISCUSSION

In Bangladesh, El Tor variant strains of V. cholerae O1 that belong to the El Tor biotype but produce cholera toxin of the classical type have completely replaced the prototype seventh pandemic El Tor strain (Nair et al., 2006). Such strains have also been identiﬁed in several other countries in Asia and Africa (Nguyen et al., 2009; Raychoudhuri et al., 2009; Safa et al., 2008). This suggests that the El Tor variants of V. cholerae O1 have now prevailed and become the epidemic strains. Cholera has become a predominantly travel-associated disease in Japan, where most cases are associated with travel to Asian countries. Accordingly, we examined V. cholerae O1 strains from travel-associated cholera cases in Japan as an indicator of the epidemiology.
in Asian endemic areas. All the 67 strains in this study were confirmed to be El Tor biotype. We typed the ctxB gene of all the biotype El Tor strains by MAMA-PCR to identify the El Tor variants of V. cholerae O1. As shown in Table 1, El Tor variants were first identified in isolates from 1993. Of the three El Tor strains of 1994, two possessed the classical-type ctxB gene, namely, they were El Tor variants; and all the El Tor strains of V. cholerae O1 after 1994 were El Tor variants. These results include a bias associated with the relatively small number of strains and reflect that the strains were only from patients not from environmental samples. But they suggest that the emergence and spread of El Tor variants took place around 1993, and that the toxin-type shift might have been completed in the latter half of the 1990s. Namely, the toxin type of the El Tor strains shifted around 1993, and El Tor variants have now completely replaced typical El Tor strains in endemic areas of Asia. This is consistent with other recent findings, indicating that the El Tor variant has spread ubiquitously (Nair et al., 2006; Nguyen et al., 2009; Raychoudhuri et al., 2009; Safa et al., 2008).

There are two hypotheses for the marked spread of El Tor variant; the first is that it might have resulted from clonal expansion of the single ancestral El Tor variant that first acquired the classical ctxB gene. The second is that it might have developed from multiclonal emergence of the El Tor variant in each region; therefore, we performed two different molecular genotyping methods, PFGE and MLVA, to examine the clonality of the El Tor variants. Both methods are accepted as those with discriminatory power and reproducibility, but their results make no obvious correlations with each other (Danin-Poleg et al., 2007). A dendrogram based on PFGE profiles with NotI digestion is shown in Fig. 1(a). Typical classical and El Tor strains were clearly divided into different clusters with similarity of less than 75 %. And the El Tor variants were assigned into the cluster of the typical El Tor strains. The biotype El Tor strains formed multiple minor clusters, some of which were related to their geographical area and/ or time, but overall they intermingled with each other. MLVA is a newly developed technique for genotyping bacterial micro-organisms based on the combination of various short tandem repeats at multiple loci. In this study, we selected seven loci that displayed a relatively high resolution power in a previous study (Danin-Poleg et al., 2007). The number of variations and Simpson's diversity index of each locus are shown in Table 2. After combining all the loci, 70 types were identified for 78 strains and the overall diversity index was 0.98, indicating that these seven loci are appropriate for molecular genotyping. Fig. 1(b) shows the dendrogram generated from the results of MLVA. As observed in Fig. 1(a), the biotype El Tor strains were not divided into obvious clusters by their toxin type.

We used two methods, PFGE and MLVA, for molecular typing to examine the bacterial genome by different criteria and to reinforce the results from each individual assay. Similar results were observed not only in PFGE analysis but also in MLVA, though the components of some of the minor clusters were different between the methods. Furthermore, even the oldest El Tor variants in this study had already showed some genetic diversity and were divided into different minor clusters. These results suggest that El Tor variants are related to various types of typical El Tor strains rather than classical type strains, and that the El Tor variant epidemic was likely to be caused by simultaneous or sequential emergence and expansion of multiclones, and not by the prevalence of a certain single clone.

In 1992, a new serogroup, defined as O139, caused a severe outbreak of cholera in South-East India. During the following 10 months, the O139 serogroup spread throughout the Indian subcontinent and soon thereafter spread to neighbouring countries, resulting in temporary displacement of the O1 serogroup (Sack et al., 2004; Bhattacharya et al., 1993). This period coincides with the emergence and spread of the El Tor variant strains. The seventh pandemic of V. cholerae O1 is ongoing after a transient epidemic due to V. cholerae O139. The re-emerged V. cholerae O1 biotype El Tor strains, however, have been reported to differ in genetic characteristics from the V. cholerae O1 biotype El Tor strains of the pre-O139 period, and identification of strains that could not be biotyped as El Tor or classical has also been reported (Nair et al., 2006; Faruque et al., 1997;

### Table 2. Primers used for MLVA and repeat variations of V. cholerae O1

<table>
<thead>
<tr>
<th>Locus name</th>
<th>Locus tag in N16961</th>
<th>Primer pair (5’→3’)</th>
<th>No. of alleles</th>
<th>Index of diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC0147</td>
<td>CTGCAACGGATACCTAACCAGGAGA</td>
<td>TTCAACATCGTGAGGTT</td>
<td>7</td>
<td>0.782</td>
</tr>
<tr>
<td>VC0436-7</td>
<td>GAGGTTAAAGCTCCTAACAAGGTTT</td>
<td>CGGCGCATCCAGCCTGAGTTT</td>
<td>5</td>
<td>0.472</td>
</tr>
<tr>
<td>VC0590</td>
<td>GTATCTCAATTGCTCTGTTGACG</td>
<td>CATGACAGTGAAACTATGAGC</td>
<td>4</td>
<td>0.337</td>
</tr>
<tr>
<td>VC1457-8</td>
<td>GCTAAAAACAGAATAATGATATAAAAGGAC</td>
<td>CATTGGTCTCCTGGTTTCAAGTACTC</td>
<td>6</td>
<td>0.467</td>
</tr>
<tr>
<td>VC1650</td>
<td>GGCTGATACATTGCAACCGAT</td>
<td>CGAATTGGCCTCACTGAGTGA</td>
<td>8</td>
<td>0.807</td>
</tr>
<tr>
<td>VCA0171</td>
<td>CGACACATTAGACCTGTTCA</td>
<td>CTGATTTCAGAGGATTTCGTAGTACG</td>
<td>19</td>
<td>0.877</td>
</tr>
<tr>
<td>VCA0283</td>
<td>AATATGCTGACCTCCTGAC</td>
<td>CTCTGAACTGGTTTGTGACG</td>
<td>20</td>
<td>0.929</td>
</tr>
</tbody>
</table>
The mechanism of emergence and the evolutionary process of the El Tor variant remain to be elucidated. More broad and extensive surveillance of clinical and environmental strains should be carried out to clarify the evolutionary process of the newly emerging type of *V. cholerae* O1. However, in this process, phage-mediated lateral gene transfer could also be involved in the acquisition of the *ctx* gene by ancestor El Tor strains from putative phage sources such as non-O1 non-O139 *V. cholerae* as suggested by Udden et al. (2008). We again emphasize that while statistically significant correlations suggest that our genotype clusters align themselves with different environmental conditions, further characterization of the sampling sites and the relative fitness of genotypes is needed to assess the importance of our observed genomic diversity.

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