Emergence of a New Clone of Toxigenic Vibrio cholerae O1 Biotype El Tor Displacing V. cholerae O139 Bengal in Bangladesh

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The emergence of Vibrio cholerae O139 Bengal in 1992, its initial rapid spread throughout Bangladesh and neighboring countries, and its propensity to replace the existing strains of V. cholerae O1 during 1992 and 1993, and the subsequent reemergence of V. cholerae O1 of the El Tor biotype in Bangladesh since 1994 have raised questions regarding the origin of the reemerged El Tor vibrios. We studied 50 El Tor vibrio strains isolated in Bangladesh and four other countries in Asia and Africa before the emergence of V. cholerae O139 and 32 strains isolated in Bangladesh during and after the epidemic caused by V. cholerae O139 to determine whether the reemerged El Tor vibrios were genetically different from the El Tor vibrios which existed before the emergence of V. cholerae O139. Analysis of restriction fragment length polymorphisms in genes for conserved rRNA, cholera toxin (ctxA), and zonula occludens toxin (zot) or in DNA sequences flanking these genes showed that the El Tor strains isolated before the emergence of V. cholerae O139 belonged to four different ribotypes and four different ctx genotypes. Of 32 El Tor strains isolated after the emergence of O139 vibrios, 30 strains (93.7%) including all the clinical isolates belonged to a single new ribotype and a distinctly different ctx genotype. These results provide evidence that the reemerged El Tor strains represent a new clone of El Tor vibrios distinctly different from the earlier clones of El Tor vibrios which were replaced by the O139 vibrios. Further analysis showed that all the strains carried the structural and regulatory genes for toxin-coregulated pilus (tcpA, tcpI, and toxR). All strains of the new clone produced cholera toxin (CT) in vitro, as assayed by the GM1-dependent enzyme-linked immunosorbent assay, and the level of CT production was comparable to that of previous epidemic isolates of El Tor vibrios. Further studies are required to assess the epidemic potential of the newly emerged clone of V. cholerae O1 and to understand the mechanism of emergence of new clones of toxigenic V. cholerae.

MATERIALS AND METHODS

V. cholerae strains. A total of 82 V. cholerae O1 isolates, obtained from cholera patients and environmental surface water either before or after the emergence of V. cholerae O139, were included in the study. The strains isolated before the emergence of V. cholerae O139 included 29 strains isolated in Bangladesh between 1969 and 1992 and 21 strains from four other countries in Asia and Africa isolated during 1991 and 1992. Clinical isolates from Bangladesh were obtained from patients who attended the treatment center of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), located in Dhaka. Other Asian isolates consisted of five El Tor strains from Syria (courtesy of F. Harb, Public Health Laboratory, Damascus, Syria) and three El Tor strains from India (courtesy of G. B. Nair, National Institute for Cholera and Enteric Diseases, Calcutta, India). The African strains consisted of seven El Tor strains from Tanzania (courtesy of F. Malu, Muhimbili Medical Centre, Dar-es-Salam, Tanzania) and six El Tor strains from Nigeria (courtesy of H. van Vliet, World Health Organization, Lagos, Nigeria). The El Tor strains isolated in Bangladesh during and after the epidemic caused by V. cholerae O139 included 32 strains, of which 27 were patient isolates and 5 were environmental isolates. Strains were stored either in lyophilized form or in sealed deep nutrient agar at room temperature in the culture collection of the ICDDR,B. Before use, the identities of the cultures were confirmed by biochemical reaction and serology (37). Details of the strains are presented in Table 1.

Preparation of colony and Southern blots. Colony blots were prepared by using nylon filters (Hybond; Amersham International plc, Aylesbury, United Kingdom) and were processed by a standard method (18). Briefly, colonies were lysed with denaturing solution (0.5 M NaOH, 1.5 M NaCl) and were neutralized in neutralizing solution (0.5 M Tris-HCl [pH 8.0], 1.5 M NaCl), and the liberated DNA was fixed to the nylon membrane by exposure to UV light for 3 min in accordance with the supplier's instructions.

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TABLE 1. Restriction endonuclease cleavage patterns of cholera toxin (ctxA), zonula occludens toxin (zot), and rRNA genes among 82 V. cholerae O1 strains of the El Tor biotype isolated before or after the emergence of V. cholerae O139*

<table>
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<tr>
<th>Strains isolated before the emergence of V. cholerae O139</th>
<th>Strains isolated after the emergence of V. cholerae O139</th>
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<tr>
<td>V. cholerae O139</td>
<td>V. cholerae O139</td>
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<tr>
<td>Bangladesh 1980 Patient 3</td>
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<tr>
<td>India 1992 Patient 3</td>
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<tr>
<td>Syria 1992 Patient 5</td>
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<td>Nigeria 1992 Patient</td>
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* The presence of tcp genes was detected by PCR assays. All strains carried tcpA (characteristic of the El Tor biotype), tcpD, and tcpF.


d and culture conditions. Statistical comparison of CT production between two groups of strains was carried out by the Mann-Whitney test, and comparison of that between more than two groups was done by the Kruskal-Wallis test. Differences were considered to be significant when P ≤0.05. Data analysis was carried out by using statistical software (Sigmasat, version 1.0., for Windows; Jandel Scientific, San Rafael, Calif.).

RESULTS

rRNA gene restriction patterns. Analysis of rRNA genes with BglI produced reproducible restriction patterns, and the 82 strains could be differentiated into five different ribotypes. The restriction patterns (Fig. 1) consisted of 7 to 10 bands between 12 and 1.6 kb in size. Of the 50 strains isolated before the emergence of V. cholerae O139, 23 (46%) belonged to ribotype I, 17 (34%) belonged to ribotype II, 3 (6%) belonged to ribotype III, and 7 (14%) belonged to ribotype IV. Of the 32 El Tor strains isolated after the emergence of V. cholerae O139, 30 strains (93%), including all 27 clinical isolates, belonged to ribotype V, and the remaining 2 environmental isolates belonged to ribotype II (Table 1). The restriction pattern representing ribotype V contained a unique band of 0.7 kb which was not present in any of the other restriction patterns (Fig. 1).

Restriction fragment length polymorphism analysis of the ctxA and zot genes. Restriction fragment length polymorphism analysis of the ctxA and zot genes with the enzyme BglI revealed five different restriction patterns for each of the ctxA (patterns A through E) and zot (patterns A through E)
The ctxA patterns consisted of one to two bands between 8.1 and 3.0 kb, and the zot patterns consisted of two to three bands between 9.2 and 3.0 kb (Fig. 2A and B). The number of bands comprising a zot restriction pattern was always one band more than the number of bands comprising the ctxA restriction pattern produced by the same strain. Four of the ctxA restriction patterns (patterns A through D) and the corresponding zot restriction patterns (patterns a through d) were shared by El Tor strains isolated before the emergence of V. cholerae O139; whereas 30 of the 32 El Tor strains isolated after the emergence of V. cholerae O139 produced restriction patterns E and E with the ctxA and the zot gene probes, respectively (Table 1). The 82 strains were differentiated into five different ctx genotypes on the basis of the BglI restriction patterns of their ctxA and zot genes.

**Analysis of tcp and toxR genes.** All strains were positive for the tcpA, tcpI, and toxR genes. PCR assay for tcpA amplified a 0.47-kb portion of the tcpA gene in all the strains (Fig. 3). This was characteristic of the El Tor biotype tcpA gene. PCR assay for the tcpI gene produced an amplicon of 2.1 kb. Subsequent restriction analysis of the amplicon with BclI, HaeIII, or XbaI produced sets of fragments whose sizes agreed with the expected sizes based on the published sequence of tcpI. Digestion with BclI produced two fragments of 1.5 and 0.6 kb, digestion with XbaI produced two fragments of 1.7 and 0.4 kb, and digestion with HaeIII produced four fragments of 0.85, 0.75, 0.27, and 0.19 kb (Fig. 4).

Colony blot hybridization revealed that all the strains in the present study carried the sequence for ToxR. Subsequent Southern blot hybridization of HindIII-digested total DNA with the toxR probe produced identical band patterns for all the strains (Fig. 5). The patterns consisted of two bands of 9.1 and 2.3 kb.

**Production of CT.** All the El Tor strains isolated after the emergence of O139 vibrios produced CT in vitro when they were cultured in AKI medium at 30°C. The level of CT production varied between 0.25 and 12.75 ng/ml among the different strains. There was no significant difference (P = 0.464) between the levels of CT produced by the El Tor strains isolated before and after the emergence of O139 vibrios (Table 2).

**DISCUSSION**

Several previous studies have demonstrated the appearance and disappearance of different clones of toxigenic V. cholerae in Bangladesh (6, 9, 11, 27, 29, 30). These include the transient appearance of multiple-drug-resistant strains (11), the disappearance and reemergence of Classical V. cholerae in Bang-
and the emergence of a non-O1 *V. cholerae* (O139) strain as the predominant epidemic strain (1, 30). Soon after the emergence of *V. cholerae* O139, the existing strains of *V. cholerae* O1 (mostly of the El Tor biotype) were almost completely displaced, possibly through a competitive mechanism which might have involved unidentified environmental factors as well as preexisting immunity in the host population. However, the gradual reemergence of El Tor strains of *V. cholerae* O1 since 1994 and the decline of the O139 strains need to be explained. The present study was designed to inquire whether the reemergence of El Tor vibrios was a result of the domination of preexisting clones of El Tor vibrios over O139 vibrios due to possible changes in environmental circumstances or whether the reemerged El Tor strains represent a new clone of toxigenic *V. cholerae* O1 which was able to compete better than the previous clones. A clone refers to bacterial isolates which share so many identical phenotypic and genetic traits that the most likely explanation is a common origin. We have previously examined the restriction patterns of conserved rRNA genes (ribotypes) and CT genes or DNA flanking these genes to differentiate among clones of toxigenic *V. cholerae* which are otherwise phenotypically identical (6, 7, 9). These studies have demonstrated that the restriction patterns are reproducible and may be considered fairly stable markers for identifying different clones.

**Clonal diversity of El Tor strains.** Clonal diversity among El Tor strains has been documented previously (9, 17, 23), and those studies suggested that toxigenic El Tor strains might have evolved from several parental strains or clones. In the present study, the 82 strains could be differentiated and were found to belong to five different ribotypes (ribotypes I through IV), whereas 93.75% (30 of 32) of the isolates obtained after the emergence of the O139 vibrios belonged to a single ribotype (ribotype V). We have also previously reported (9) the rRNA gene restriction patterns corresponding to ribotypes I through IV found in the present study. However, cleavage pattern V produced by the post-O139 strains in this study have not been reported previously by us or other investigators who have analyzed a large number of El Tor, Classical, and O139 strains from different countries (7, 17, 23). This suggests that the post-O139 El Tor strains represent a new clone of El Tor vibrios. Although all the El Tor vibrios isolated from patients and environmental surface water from 1994 to 1996 belonged to this new ribotype, two strains isolated from environmental surface water during 1993 and 1994 belonged to ribotype II. These two isolates may have been remnants of the El Tor strains which were being replaced by *V. cholerae* O139.

Probing of the *Bgl*I restriction fragments of the chromosome for the *ctxA* and *zot* genes also revealed differences among the El Tor vibrios isolated before and after the emergence of the O139 vibrios. In *V. cholerae*, the genes encoding cholera toxin (*ctxAB*) and zonula occludens toxin (*zot*) are part of a larger...
genetic element (ctx genetic element) consisting of at least five genes (comprising the core region) that is flanked by two or more copies of a repeated sequence (22, 35). Although there is very little variation among the structural sequences of CTs from different strains, restriction fragment length polymorphism in ctx is observed due to variation in the number of copies of the ctx genetic element carried by different strains as well as variation in the chromosomal sequence flanking the ctx element. In the present study the restriction endonuclease used was BglI, which does not have any recognition sequence within the ctx gene, but it has a single cleavage site within the zot gene located upstream and adjacent to the ctxA gene (3, 19). Consequently, the number of bands comprising each ctxA restriction pattern represented the possible numbers of copies of the ctx element carried by the strain. The number of bands comprising the zot restriction patterns was one band more than the number of bands comprising the ctx restriction pattern in the same strain (Fig. 2A and B), suggesting that strains carrying more than one copy of the ctx element possibly had the copies located adjacent to each other in the chromosome. On the basis of the ctxA and zot restriction patterns, the strains were grouped into five different ctx genotypes (Table 1). While ctx genotypes 1 through 4 were shared by the 50 El Tor strains isolated before the emergence of O139 vibrios, 30 of the 32 El Tor strains from the post-O139 period belonged to ctx genotype 5. All 30 strains belonged to the new ribotype (ribotype V). The ctxA restriction patterns of the 30 isolates belonging to the new ribotype were also different from our previously reported restriction patterns of ctxA or its flanking DNA sequences in Classical, El Tor, or O139 vibrios (7). Hence, the ribotype data and the ctx genotype data agreed, providing further evidence that the post-O139 El Tor vibrios isolated in Bangladesh represent a new clone.

**Analysis of tcp and toxR genes.** Colonization of brush borders in the small intestine, a crucial component of the infection strategy of *V. cholerae*, is assumed to be mediated by a rigid pilus colonization factor designated toxin-coregulated pilus (TCP), since it is under the same genetic control as CT, and involves the ToxR-ToxT regulatory cascade (12, 21, 34). Molecular analysis has revealed that although the major subunit of TCP is TcpA, the formation and function of the pilus assembly require the products of a number of other genes located on the chromosome adjacent to the tcpA gene, and these constitute a tcp gene cluster (21). The tcpH and tcpI genes are two *ToxR*-regulated genes that affect TcpA synthesis. It has been suggested that regulators such as TcpI that act downstream of *ToxR* and *ToxT* may function to fine-tune the expression of the TCP virulence determinant throughout the pathogenic cycle of *V. cholerae* (12).

In the present study all the El Tor strains carried the tcpA, tcpI, and toxR genes. Although the post-O139 El Tor vibrios were different from the El Tor vibrios isolated before the emergence of *V. cholerae* O139 in terms of RNA, ctxA, and zot restriction patterns, restriction analysis of the PCR-amplified tcpI gene and Southern blot hybridization of the toxR gene showed that both of these regulatory genes are highly conserved among the 82 El Tor strains studied. A recent report by Waldor and Mekalanos (36) suggested that lysogenic conversion by a bacteriophage designated ctxΦ encoding CT can give rise to toxigenic strains from nontoxigenic *V. cholerae* strains and that the phage conversion requires expression of TCP, which is used as a receptor by the bacteriophage. Hence, the possibility that the new clone of El Tor vibrios arose as a consequence of bacteriophage conversion of a nontoxigenic strain of *V. cholerae* O1 cannot be ruled out. Furthermore, integration of the phage genome (the ctx genetic element) into the host chromosome at particular sites is specified by the presence of a 17-bp sequence called attRS1 (22, 36). The distinct ctxA restriction pattern produced by the new clone of El Tor vibrios suggests that integration of the ctx genetic element in these strains might have occurred at chromosomal sites different from those for the other El Tor vibrios, possibly due to the presence of the attRS1 sequence in these sites of the nontoxigenic parental strain. However, further

<table>
<thead>
<tr>
<th>Strain and country of origin</th>
<th>Yr of isolation</th>
<th>Source</th>
<th>No. of isolates</th>
<th>ctx genotype</th>
<th>No. of ctx copies</th>
<th>CT production (concn [ng/ml])^a^</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bangladesh</td>
<td>1970–1976</td>
<td>Patient</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>4.84</td>
<td>2.39–6.37</td>
<td></td>
</tr>
<tr>
<td>Bangladesh</td>
<td>1969–1976</td>
<td>Patient</td>
<td>7</td>
<td>2</td>
<td>2</td>
<td>3.96</td>
<td>1.32–8.67</td>
<td></td>
</tr>
<tr>
<td>Bangladesh</td>
<td>1991–1992</td>
<td>S. water</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>2.84</td>
<td>1.36–3.52</td>
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</tr>
<tr>
<td>Bangladesh</td>
<td>1991–1992</td>
<td>Patient</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>2.72</td>
<td>1.12–3.82</td>
<td></td>
</tr>
<tr>
<td>Bangladesh</td>
<td>1990</td>
<td>Patient</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>2.45</td>
<td>1.75–3.75</td>
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</tr>
<tr>
<td>India</td>
<td>1992</td>
<td>Patient</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>3.61</td>
<td>2.73–5.58</td>
<td></td>
</tr>
<tr>
<td>Syria</td>
<td>1992</td>
<td>Patient</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>3.23</td>
<td>1.50–4.34</td>
<td></td>
</tr>
<tr>
<td>Nigeria</td>
<td>1992</td>
<td>Patient</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>3.93</td>
<td>3.52–7.87</td>
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<th>Strains isolated after the emergence of <em>V. cholerae</em> O139</th>
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<td>Bangladesh</td>
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<td>Bangladesh</td>
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<td>Bangladesh</td>
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</table>

^a^ Differences in the median values of CT produced by different groups of strains were not statistically significant (P = 0.003). The difference in the median concentrations of CT produced by the 50 El Tor strains isolated before and the 32 El Tor strains isolated after the emergence of *V. cholerae* O139 was also statistically insignificant (P = 0.464). 

^b^ S. water, surface water.
studies and identification of the possible nontoxicogenic parental strain is essential to confirm these assumptions. An alternative explanation could, however, be that strains belonging to the new clone existed in the environment in very low numbers and hence were not detected in the past, but that some unidentified environmental changes have caused these strains to multiply rapidly and to become dominant over existing strains of V. cholerae O139. It is interesting that in many parts of neighboring India, as in Bangladesh, after the initial dominance of V. cholerae O139, it has been replaced by El Tor vibrios (2).

**Epidemic potential of the new clone of El Tor vibrios.** The mechanism involved in the domination of a newly emerged clone of toxicogenic V. cholerae resulting in the displacement of existing clones is not clear, although unidentified environmental factors are likely to influence the process. Previous examples of the emergence or reemergence of different clones of toxicogenic V. cholerae were often associated with epidemic outbreaks of cholera caused by the newly emerged strain (1, 6, 11, 27). All the strains examined in the present study were isolated from the capital city of Bangladesh, Dhaka, which is overcrowded, and the growth in population has outstripped the capacity to provide adequate housing and sanitation facilities. The capital is surrounded by large semirural population centers and receives an enormous influx of people from rural villages and hence serves as a catchment area for representative V. cholerae strains found throughout the country. The epidemic potential of the new clone examined in this study is still not clear, but recent surveillance results for Dhaka and several rural districts of Bangladesh and results of molecular analysis of strains isolated in Dhaka suggest that the El Tor vibrios which have already replaced the O139 vibrios, at least in the northern and central parts of Bangladesh, may also belong to the new clone. Studies are under way to collect and analyze strains from different rural areas in Bangladesh.

It has been suggested that CT, TCP, and the ToxR regulon are essential for V. cholerae pathogenesis in humans (13). In the present study, all the El Tor strains belonging to the new clone carried the genes for tcpA, tcpI, ctxA, and zot and, presumably, the complete ctx genetic element and the tcp gene clusters, in addition to the toxR gene. Most of these El Tor vibrios were isolated from cholera patients, and the strains produced CT in vitro, the level of which was comparable to that produced by strains isolated from previous cholera epidemics. Hence, the possibility that the new clone can give rise to spreading outbreaks of cholera under appropriate circumstances cannot be ruled out. The movement of V. cholerae strains belonging to the new clone should therefore be carefully monitored through environmental and epidemiological surveillance. Further molecular studies are also required to understand the genetic basis of the apparent ability of the clone to compete with V. cholerae O139 better than the previously existing clones of El Tor vibrios.

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