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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 rapid spread in an epidemic form, in which it replaced 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 G_{M1}-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*.

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 led experts to suspect *V. cholerae* O139 as the new pandemic strain of cholera (1, 14, 24, 25, 30). The epidemic caused by *V. cholerae* O139 moved quickly and affected the entire coastal and estuarine tidal plains of southern Bangladesh. By the end of March 1993, the epidemic reached the middle and northern parts of the country and the coastal areas and spread eastward (1, 30). In the beginning the new strain totally displaced the existing *V. cholerae* O1 strains, including both Classical and El Tor biotypes, which coexisted only in Bangladesh. Nearly 2 years after the initial detection of O139 vibrios, striking differences in the distribution of O139 and O1 vibrios were observed. In most northern and central areas of Bangladesh, including the capital city, Dhaka, the O139 vibrios have been replaced by *V. cholerae* O1 of the El Tor biotype (5, 28). In the southern coastal regions, however, the O139 vibrios continue to exist (28).

The factors which determine the emergence and domination of particular clones of toxigenic *V. cholerae* through natural selection are not clear. The disappearance and reemergence of the El Tor biotype of *V. cholerae* O1 in Bangladesh have raised questions regarding the possible origin of the reemerged El Tor strains. In the present study we have used molecular techniques to analyze El Tor strains collected before and after the emergence of *V. cholerae* O139 to investigate whether the El Tor strains isolated before the emergence of O139 vibrios were genetically different from the El Tor strains isolated after the epidemic caused by *V. cholerae* O139.

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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. Mahlu, 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, Ayelesbury, 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.

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[a]

Strains and country of origin	Yr of isolation	Source	No. of isolates	*Bgl*I cleavage pattern of genes			Designated *ctx* genotype[b]
				ctxA	*zot*	rRNA	
Strains isolated before the emergence of *V. cholerae* O139							
Bangladesh	1970–1976	Patient	5	A	a	I	1
Bangladesh	1969–1976	Patient	7	B	b	I	2
Bangladesh	1991–1992	S. water[c]	5	B	b	I	2
Bangladesh	1991–1992	Patient	6	C	c	II	3
Bangladesh	1991–1992	S. water	3	C	c	II	3
Bangladesh	1990	Patient	3	D	d	III	4
India	1992	Patient	3	B	b	I	2
Syria	1992	Patient	5	C	c	II	3
Nigeria	1992	Patient	6	B	b	I	2
Tanzania	1991–1992	Patient	7	B	b	IV	2
Strains isolated after the emergence of *V. cholerae* O139							
Bangladesh	1993–1994	S. water	2	C	c	II	3
Bangladesh	1994–1995	S. water	3	E	e	V	5
Bangladesh	1995	Patient	16	E	e	V	5
Bangladesh	1996	Patient	11	E	e	V	5

[a] The presence of *tcp* genes was detected by PCR assays. All strains carried *tcpA* (characteristic of the El Tor biotype), *tcpI*, and *toxR*.
[b] Genotype based on restriction patterns of *ctxA* and *zot* genes.
[c] S. water, surface water.

For preparation of DNA blots, total cellular DNA was isolated from overnight cultures as described previously (32). Five-microgram aliquots of the DNA were digested with appropriate restriction enzymes (Bethesda Research Laboratories, Gaithersburg, Md.), electrophoresed in 0.8% agarose gels, and blotted onto nylon membranes (Hybond; Amersham) by Southern blotting (31).

Probes and hybridization. The rRNA gene probe was a 7.5-kb *Bam*HI fragment of pKK3535 (4), which is a pBR322-derived plasmid containing an *Escherichia coli* rRNA operon consisting of one copy each of the genes coding for 5S rRNA, 16S rRNA, 23S rRNA, and $tRNA^{Glu}$. The gene probe for cholera toxin (CT) was a 0.5-kb *Eco*RI fragment of pCVD27 (15), which is a pBR325-derived plasmid containing an *Xba*I-*Cla*I fragment representing 94% of the gene encoding the A subunit of CT (*ctxA*) cloned with *Eco*RI linkers. The probe for zonula occludens toxin (Zot) was an 850-bp region internal to the *zot* gene and was amplified from the recombinant plasmid pBB241 (3) as we have described previously (8). The *toxR* gene probe was a 2.4-kb *Bam*HI fragment of pVM7 (20), which is a pBR322-derived plasmid carrying the entire *toxR* sequence.

The probes used for hybridization were labelled by random priming (10) by using a random primer DNA labelling kit (Bethesda Research Laboratories) and [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham). Southern blots and colony blots were prehybridized and hybridized with the labelled probes at 68°C as described previously (9). Hybridized blots were washed once in 2× SSC (1× SSC is 0.15 M NaCl plus 0.15 M sodium citrate) for 5 min at room temperature, two times in 2× SSC–0.1% sodium dodecyl sulfate for 10 min at 68°C, and once in 0.1× SSC–0.1% sodium dodecyl sulfate for 15 min at 68°C. Autoradiographs were developed from the hybridized filters by using either Kodak X-Omat AR film (Kodak, Rochester, N.Y.) or Fuji X-ray film at −70°C as described previously (9).

PCR assays. The presence of *tcpA* genes specific for the Classical and El Tor biotypes was determined by a multiplex PCR assay as described previously (14). The *tcpI* gene was detected by a PCR assay based on the published sequence of *tcpI* (12), as follows. Two primers, 5′-AACGATAAAGCTGATTTTCAC and 5′-CCAAGGCTTAGAGCCTTTTAT, were synthesized commercially by Oswel DNA Service (University of Edinburgh, Edinburgh, United Kingdom). Thermocycle parameters for the PCR assay consisted of denaturation at 94°C for 2 min, annealing of primers at 50°C for 2 min, and primer extension at 72°C for 3 min. Amplification was performed for 25 cycles, and the expected size of the amplicon (2.1 kb) was ascertained by electrophoresis in 1.5% agarose gels. The identity of the PCR product was further verified by digesting the PCR product with the restriction enzymes *Bcl*I, *Hae*III, or *Xba*I and determining the sizes of the digestion fragments by agarose gel electrophoresis and comparing the sizes of the fragments with expected sizes based on the published sequence of the *tcpI* gene (12).

Assay for CT production. The ability of the strains to produce CT in vitro was determined by the G_{M1}-ganglioside-dependent enzyme-linked immunosorbent assay (G_{M1}-ELISA) as described previously (26, 33). For each round of the CT assay, 5 ml of AKI medium (1.5% Bacto Peptone, 0.4% yeast extract, 0.5% NaCl, 0.3% $NaHCO_3$ [pH 7.4]) was inoculated with approximately 10^3 bacterial cells, and the cells were grown for 16 h at 30°C with shaking. The culture was centrifuged at 4,000 × *g* for 5 min, and the supernatant was collected. Aliquots of the undiluted supernatant, 10-fold and 100-fold dilutions of the supernatant, and dilutions of purified CT (Sigma Chemical Company, St. Louis, Mo.) were used for the toxin assay. Briefly, 100 μl of the samples was added to each well of microtiter plates precoated with G_{M1}, and the plates were incubated at room temperature for 90 min. After washing the plates with phosphate-buffered saline containing 0.5% Tween 20, the G_{M1}-bound CT was reacted with rabbit anti-CT monoclonal antibody (Sigma). Antibody binding to CT was detected by reaction with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (whole molecule) antibody (Sigma) and the substrates *o*-phenyldiamine and hydrogen peroxide. Quantitation of CT production was done by using a standard curve prepared for each batch of the assay mixture. The amount of CT produced by each strain was the mean value of three different assays with the same strain 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 was ≤0.05. Data analysis was carried out by using statistical software (Sigmastat, version 1.0, for Windows; Jandel Scientific, San Rafael, Calif.).

RESULTS

rRNA gene restriction patterns. Analysis of rRNA genes with *Bgl*I 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 (93.7%), 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 8.7 kb which was not present in any of the other restriction patterns (Fig. 1).

Restriction patterns of *ctxA* and *zot* genes. Restriction fragment length polymorphism analysis of the *ctxA* and *zot* genes with the enzyme *Bgl*I revealed five different restriction patterns for each of the *ctxA* (patterns A through E) and *zot* (patterns

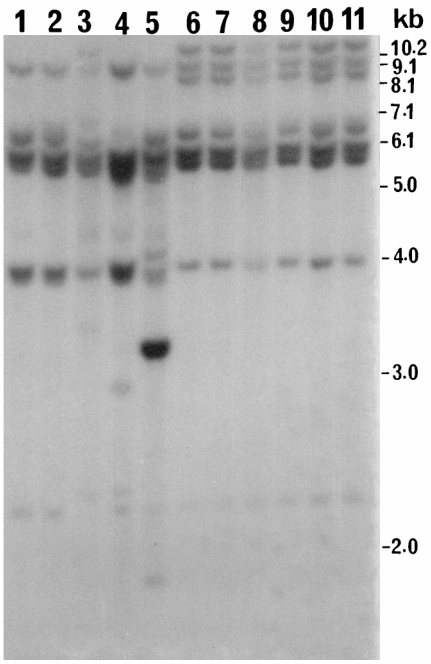

FIG. 1. Southern hybridization analysis of genomic DNA from *V. cholerae* digested with *Bgl*I and probed with a 7.5-kb *Bam*HI fragment of the *E. coli* rRNA clone pKK3535. Lanes 1 and 2, *Bgl*I restriction pattern I; lanes 3 through 5, restriction patterns II through IV, respectively, produced by El Tor vibrios isolated before the emergence of *V. cholerae* O139; lanes 6 through 11, *Bgl*I restriction pattern V produced by El Tor strains isolated after the emergence of *V. cholerae* O139. Numbers indicate the molecular sizes of bands corresponding to a 1-kb DNA ladder (Bethesda Research Laboratories), used as a molecular size marker.

a through e) genes. 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* gene 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 O139 vibrios 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 *Bgl*I 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 *Bcl*I, *Hae*III, or *Xba*I produced sets of fragments whose sizes agreed with the expected sizes based on the published sequence of *tcpI*. Digestion with *Bcl*I produced two fragments of 1.5 and 0.6 kb, digestion with *Xba*I produced two fragments of 1.7 and 0.4 kb, and digestion with *Hae*III 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 *Hin*dIII-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-

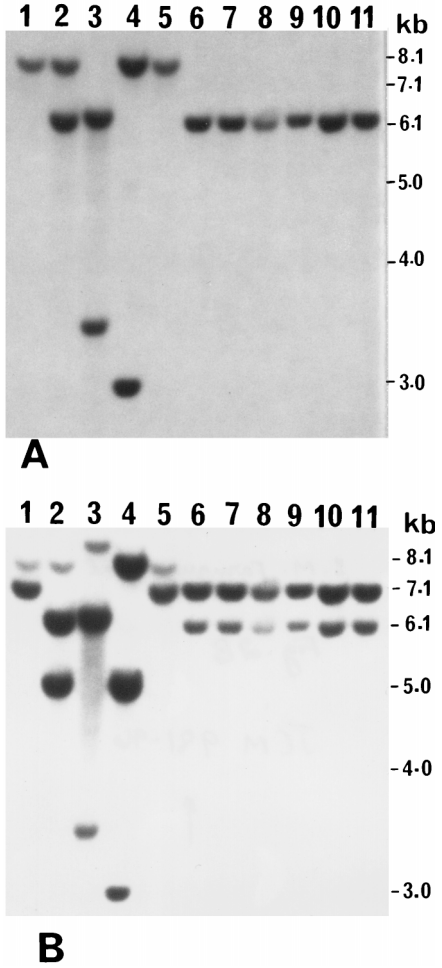

FIG. 2. Southern hybridization analysis of genomic DNA from *V. cholerae* digested with *Bgl*I and probed with a 550-bp fragment of the A subunit of the CT gene (A) and with a 850-bp PCR-generated probe for the zonula occludens toxin gene (B). *Bgl*I restriction pattern I (lanes 1 and 5 in Fig. 1A and B) and patterns II through IV (lanes 2 through 4, respectively, in Fig. 1A and B) correspond to *ctx* genotypes 1 and 2 through 4, respectively, demonstrated by El Tor strains isolated before the emergence of *V. cholerae* O139. Restriction pattern V (lanes 6 through 11 in Fig. 1A and B) corresponds to *ctx* genotype 5 demonstrated by El Tor vibrios isolated after the emergence of *V. cholerae* O139. Numbers indicate the molecular sizes of bands corresponding to a 1-kb DNA ladder (Bethesda Research Laboratories).

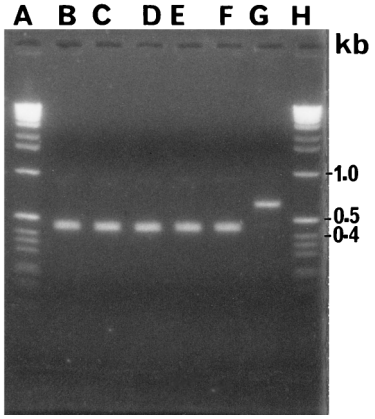

FIG. 3. Agarose gel electrophoresis of PCR products derived from multiplex PCR assays with El Tor and Classical biotype-specific primers for the *tcpA* gene. Lanes B and C, products from two El Tor strains isolated before the emergence of *V. cholerae* O139; lanes D and E, PCR products from two strains isolated after the emergence of *V. cholerae* O139; lanes F and G, control El Tor and Classical strains, respectively. Numbers indicate the molecular sizes of bands corresponding to low-molecular-weight fragments of a 1-kb DNA ladder (Bethesda Research Laboratories) (lanes A and H).

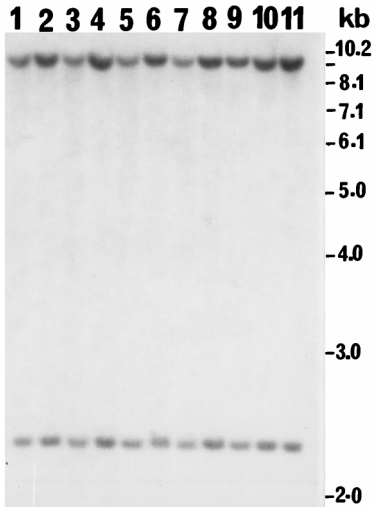

FIG. 5. Southern hybridization analysis of genomic DNA from *V. cholerae* digested with *Hin*dIII and probed with the *toxR* gene probe. Lanes 1 through 5, strains isolated before the emergence of *V. cholerae* O139; lanes 6 through 11, strains isolated after the emergence of *V. cholerae* O139. Numbers indicate the molecular sizes of bands corresponding to a 1-kb DNA ladder (Bethesda Research Laboratories).

ladesh (6, 27, 29), 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.

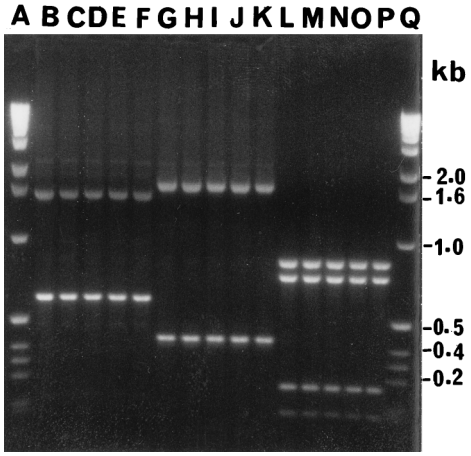

FIG. 4. Restriction analysis of a 2.1-kb PCR-generated fragment of *tcpI* gene from El Tor strains isolated before (lanes B, C, G, H, L, and M) and after (lanes D through F, H through J, and N through P) the emergence of *V. cholerae* O139. Cleavage patterns with *Bcl*I (lanes B through F), *Xba*I (lanes G through K), and *Hae*III (lanes L through P) are shown. Numbers indicate the molecular sizes of bands corresponding to low-molecular-weight fragments of a 1-kb DNA ladder (Bethesda Research Laboratories) (lanes A and Q).

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 on the basis of the *Bgl*I cleavage patterns of their rRNA genes (Fig. 1). The El Tor vibrios isolated before the emergence of O139 vibrios comprised four 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

TABLE 2. CT production by 50 El Tor vibrio strains isolated before the emergence of O139 vibrios and 32 El Tor strains isolated after the emergence of *V. cholerae* O139

Strain and country of origin	Yr of isolation	Source	No. of isolates	*ctx* genotype	No. of *ctx* copies	CT production (concn [ng/ml])[a]	
						Median	Range
Strains isolated before the emergence of *V. cholerae* O139							
Bangladesh	1970–1976	Patient	5	1	1	4.84	2.39–6.37
Bangladesh	1969–1976	Patient	7	2	2	3.96	1.32–8.67
Bangladesh	1991–1992	S. water[b]	5	2	2	2.84	1.36–3.52
Bangladesh	1991–1992	Patient	6	3	2	2.72	1.12–3.82
Bangladesh	1991–1992	S. water	3	3	2	2.12	1.51–3.17
Bangladesh	1990	Patient	3	4	2	2.45	1.75–3.75
India	1992	Patient	3	2	2	3.61	2.73–5.58
Syria	1992	Patient	5	3	2	3.23	1.50–4.34
Nigeria	1992	Patient	6	2	2	3.93	3.52–7.87
Tanzania	1991–1992	Patient	7	2	1	5.81	3.99–9.64
Strains isolated after the emergence of *V. cholerae* O139							
Bangladesh	1993–1994	S. water	2	3	2	4.72	2.95–6.49
Bangladesh	1994–1995	S. water	3	5	2	2.95	1.92–3.49
Bangladesh	1995–1996	Patient	27	5	2	3.95	0.25–12.57

[a] Differences in the median values of CT produced by different groups of strains were not statistically significant ($P = 0.063$). 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.

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 *Bgl*I, which does not have any recognition sequence within the *ctxA* 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 number 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 rRNA, *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 distinctly different *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

studies and identification of the possible nontoxigenic 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 toxigenic *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 toxigenic *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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