

Molecular Analysis of Toxigenic *Vibrio cholerae* O139 Bengal Strains Isolated in Bangladesh between 1993 and 1996: Evidence for Emergence of a New Clone of the Bengal Vibrios

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Received 19 March 1997/Returned for modification 17 May 1997/Accepted 13 June 1997

Vibrio cholerae O139 Bengal emerged in 1992 and rapidly spread in an epidemic form, in which it replaced existing strains of *V. cholerae* O1 in Bangladesh during 1992 and 1993. The subsequent emergence of a new clone of *V. cholerae* O1 of the El Tor biotype that transiently displaced the O139 vibrios during 1994 to 1995 and the recent reemergence of *V. cholerae* O139 and its coexistence with the El Tor vibrios demonstrated temporal changes in the epidemiology of cholera in Bangladesh. We studied clonal diversity among *V. cholerae* O139 strains isolated from cholera patients and environmental surface water since their first appearance until their transient disappearance in 1994 as well as the O139 strains that reemerged during 1995 to 1996 and were isolated in the capital Dhaka and four rural districts of Bangladesh to investigate the origin of the reemerged strains. Analysis of restriction fragment length polymorphisms in genes for conserved rRNA and cholera toxin (CT) (*ctxA*) or in DNA sequences flanking these genes revealed four different ribotypes and four different *ctx* genotypes among the 93 strains of *V. cholerae* O139 studied. Ribotypes I and II and *ctx* genotypes A through C were shared by strains isolated from the epidemic outbreak during 1992 and 1993 in Bangladesh and India, ribotype III was represented by a single CT-negative O139 strain from Argentina, and 16 of 27 (59.2%) of the reemerged strains isolated during 1995 and 1996 belonged to a new ribotype of O139 vibrios designated ribotype IV. All 16 strains belonging to ribotype IV also belonged to a new *ctx* genotype (genotype 4). These results provide evidence for the emergence of a new clone of toxigenic *V. cholerae* O139 in Bangladesh. Further analysis of the *rfb* gene cluster by PCR revealed the absence of a large region of the O1-specific *rfb* operon and the presence of an O139-specific genomic region in all O139 strains. The PCR amplicon corresponding to the *rfaD* gene of a CT-negative O139 strain from Argentina was smaller in length than those of the toxigenic O139 strains but was identical to those of seven non-O1 and non-O139 strains. All O139 strains except the CT-negative strain carried structural and regulatory genes for CT and toxin-coregulated pili (*ctxA*, *tcpA*, *tcpI*, and *toxR*). These results suggest that the O139 Bengal strains possibly emerged from an El Tor strain but that the CT-negative non-Bengal O139 strain might have emerged from a non-O1, non-O139 strain. Thus, strains belonging to the O139 serogroup may have emerged from similar serotype-specific genetic changes in more than one progenitor strain of *V. cholerae*.

Vibrio cholerae belonging to serogroup O1 had been considered the only causative agent of epidemic cholera until the emergence of *V. cholerae* O139 Bengal in 1992, which caused an explosive epidemic throughout Bangladesh, India, and neighboring countries (1, 29, 30, 32). In the beginning, the new strain totally displaced the existing *V. cholerae* O1 strains, including both the classical and the El Tor biotypes, which coexisted only in Bangladesh. However, during 1994 and up to the middle of 1995, in most northern and central areas of Bangladesh, including the capital city Dhaka, the O139 vibrios were replaced by a new clone of *V. cholerae* O1 of the El Tor biotype whereas in the southern coastal regions, the O139 vibrios continued to exist (6, 7, 32). During the second half of 1995 and in 1996, nearly 4 years after the initial detection of O139 vibrios, cases due to both *V. cholerae* O1 and *V. cholerae* O139 were detected in various regions of Bangladesh. Recent surveillance by the Epidemic Control Preparedness Pro-

gramme (ECPP) and Dhaka hospital surveillance of patients in the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), revealed the coexistence of *V. cholerae* O1 and O139 in Dhaka as well as in at least 4 of 10 rural districts of the northern, central, and southern regions of the country affected by outbreaks of cholera between June and December of 1996. In two of these areas, *V. cholerae* O139 was detected for the first time (details of the surveillance will be published elsewhere). In keeping with the observation in Bangladesh, in adjoining Calcutta, India, a resurgence of *V. cholerae* O139 infection overtaking *V. cholerae* O1 infection for the first time since its initial predominance in 1993 has been reported (22).

The factors which determine the emergence, disappearance, or continued existence of particular clones of toxigenic *V. cholerae* through natural selection are not clear. The transient disappearance and reemergence of the O139 vibrios in Bangladesh have raised questions regarding the possible origin of the O139 strains that have reemerged as well as possible clonal diversity among the strains belonging to the O139 serogroup. In this study, we used molecular techniques to analyze *V. cholerae* O139 collected since its initial outbreak in 1993 and its

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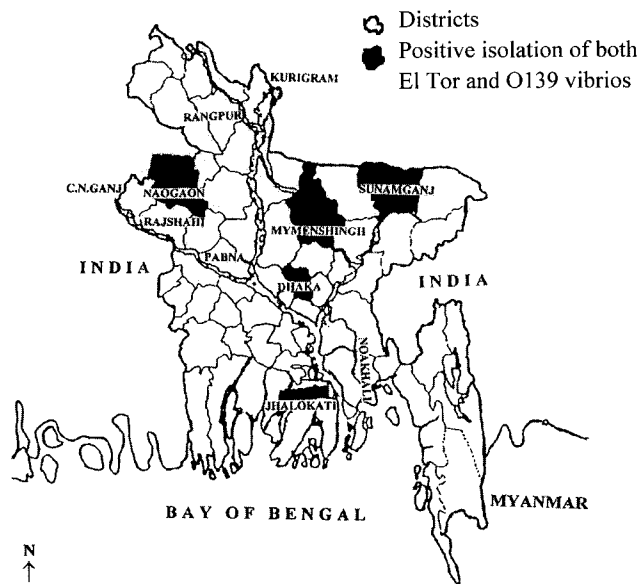


FIG. 1. Map of Bangladesh showing areas where cholera outbreaks associated with both *V. cholerae* O1 and *V. cholerae* O139 occurred between June and December of 1996.

reemergence during 1995 to 1996, as well as El Tor vibrios coexisting with *V. cholerae* O139, to study clonal relationships among the strains and to further understand the possible mechanism that led to the emergence of *V. cholerae* belonging to the O139 serogroup.

MATERIALS AND METHODS

***V. cholerae* strains.** A total of 93 *V. cholerae* O139 isolates, obtained from cholera patients and environmental surface water between 1993 and 1996, and 24 *V. cholerae* O1 isolates of the El Tor biotype, isolated from patients during 1995 and 1996 in Bangladesh, were included in the study. Clinical isolates from Bangladesh either were obtained from patients who attended the treatment center of the ICDDR,B located in Dhaka or were brought back from rural epidemic areas (Fig. 1) by the physicians of the ECPP. Seven strains of O139 vibrios isolated in India in 1992, a cholera toxin (CT)-negative O139 strain isolated from a patient in Argentina in 1993, and seven environmental isolates of *V. cholerae* that were non-O1 and non-O139 strains were also included in the study. 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 (41). Details of the strains are presented in Table 1.

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

TABLE 1. Analysis of genes for rRNA, CT (*ctxA*), toxin-coregulated pili (*tcpA* and *tcpI*), and *toxR* in 117 strains of *V. cholerae* O139 or O1 isolated between 1993 and 1996

Strain	Yr(s) of isolation	Place of isolation	Country	Source	No. of isolates	Presence of gene ^a :			rRNA gene restriction pattern ^b	<i>ctxA</i> genotype
						<i>tcpA</i>	<i>tcpI</i>	<i>toxR</i>		
<i>V. cholerae</i> O139	1993	Dhaka	Bangladesh	Patient	15	+	+	+	I	A
	1993	Dhaka	Bangladesh	Patient	11	+	+	+	I	B
	1993	Dhaka	Bangladesh	Patient	9	+	+	+	II	A
	1993	Dhaka	Bangladesh	Patient	10	+	+	+	II	C
	1993	Matlab	Bangladesh	Patient	2	+	+	+	II	C
	1992	Calcutta	India	Patient	2	+	+	+	I	A
	1992	Madras	India	Patient	2	+	+	+	II	C
	1992	Madurai	India	Patient	1	+	+	+	II	C
	1992	Vellore	India	Patient	2	+	+	+	II	C
	1993	Salta	Argentina	Patient	1	-	-	-	III	Negative
	1993	Dhaka	Bangladesh	S. water ^c	4	+	+	+	I	A
	1993	Dhaka	Bangladesh	S. water	3	+	+	+	II	C
	1993	Matlab	Bangladesh	S. water	2	+	+	+	II	C
	1994	Dhaka	Bangladesh	S. water	2	+	+	+	II	C
	1995-1996	Dhaka	Bangladesh	Patient	7	+	+	+	II	C
	1995	Dhaka	Bangladesh	S. water	2	+	+	+	IV	D
	1995-1996	Dhaka	Bangladesh	Patient	6	+	+	+	IV	D
	1996	Sunamganj	Bangladesh	Patient	2	+	+	+	II	C
	1996	Sunamganj	Bangladesh	Patient	2	+	+	+	IV	D
	1996	Naogaon	Bangladesh	Patient	4	+	+	+	IV	D
1996	Mymensingh	Bangladesh	Patient	2	+	+	+	IV	D	
1996	Jhalokati	Bangladesh	Patient	2	+	+	+	II	C	
<i>V. cholerae</i> O1, biotype El Tor	1995-1996	Dhaka	Bangladesh	Patient	3	+	+	+	V	E
	1996	Dhaka	Bangladesh	Patient	1	+	+	+	V	F
	1996	Kurigram	Bangladesh	Patient	2	+	+	+	V	E
	1996	Mymensingh	Bangladesh	Patient	2	+	+	+	V	F
	1996	Sunamganj	Bangladesh	Patient	2	+	+	+	V	E
	1996	Sunamganj	Bangladesh	Patient	2	+	+	+	V	F
	1996	Noakhali	Bangladesh	Patient	2	+	+	+	V	E
	1996	Noakhali	Bangladesh	Patient	5	+	+	+	V	F
	1996	Jhalokati	Bangladesh	Patient	5	+	+	+	V	E

^a The presence of *tcp* genes was detected by PCR assays. All strains, including *V. cholerae* O139, which carried *tcpA* produced PCR amplicons characteristic of the El Tor biotype.

^b Ribotypes and *ctxA* genotypes are based on *Bgl*I restriction patterns of the respective genes.

^c S. water, surface water.

TABLE 2. Primers used for PCR analysis of the *rfb* gene cluster

Primer	Sequence	Corresponding position of the <i>V. cholerae</i> O1 <i>rfb</i> gene cluster	Region of gene or ORF	Size of amplicon (bp)
1a	GGATAGGGCCATCAAATAT	55–74		
1b	GAACCTCAAACGTGATTTCG	552–533	<i>rfaD</i>	497
2a	TTTGAAGGATGGCGTTTAA	4273–4291		
2b	TTATTGCTTGAGAATCGCC	7072–7054	<i>rfbDEG</i>	2,799
3a	CCAGTATTATTGGCGACTTTT	14031–14033		
3b	TTGGAACATCGACTTATTGAA	14915–14895	<i>rfbNO</i>	884
4a	AGATGTAAAAGGCTGCTTGAT	16373–16393		
4b	ACGTTTGAGCTTCCAATTCTT	16767–16747	<i>rfbQRS</i>	394
5a	ATAGCGATGTGCTGTGAATT	18136–18155		
5b	CACGGAACCTTGATGTATGCT	19298–19279	<i>ompX</i>	1,162
6a	TCGACGATTTTACTGGTTC	19515–19534		
6b	CAGGAATTACAAGCGATCAA	20101–20082	<i>orf2-orf3</i>	586

For preparation of DNA blots, total cellular DNA was isolated from overnight cultures as described previously (37). 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 (34).

Probes and hybridization. The rRNA gene probe was a 7.5-kb *Bam*HI fragment of pKK3535 (3), 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 tRNAGlu. The gene probe for CT was a 0.5-kb *Eco*RI fragment of pCVD27 (15), which is a pBR325-derived plasmid containing an *Xba*I-*Cl*aI fragment representing 94% of the gene encoding the A subunit of CT (*ctxA*) cloned with *Eco*RI linkers. The *toxR* gene probe was a 2.4-kb *Bam*HI fragment of pVM7 (21), which is a pBR322-derived plasmid carrying the entire *toxR* sequence. The O139-specific DNA probe 2R3 was a 1.3-kb *Eco*RI fragment of pCRII-A3 that has been described previously (23). PCR-generated amplicons of defined regions of the *rfb* gene cluster (Table 2) of *V. cholerae* O1 strain 569B were also used as probes for different *rfb* genes.

The probes used for hybridization were labelled by random priming (11) with a random primer DNA labelling kit (Bethesda Research Laboratories) and [α -³²P]deoxycytidine triphosphate (3,000 Ci/mmol; Amersham). Southern blots and colony blots were prehybridized and hybridized with the labelled probes at 68°C as described previously (10). 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 with either Kodak X-Omat AR film (Kodak, Rochester, N.Y.) or Fuji X-ray film at –70°C as described previously (10).

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 (16). The *tcpI* gene was detected by a PCR assay based on the published sequence of *tcpI* (13) as described by us previously (7). PCR assays for the defined regions of the *rfb* gene cluster and adjoining sequences were performed with six different sets of primers (Table 2) derived from the sequence of the *V. cholerae* O1 *rfb* gene cluster (35) as described previously (2). All primers were synthesized commercially by Oswel DNA Service (University of Edinburgh, Edinburgh, United Kingdom). The expected sizes of the amplicons (Table 2) were ascertained by electrophoresis in agarose gels, and the identity of each PCR product was further verified by Southern blot hybridization (34).

RESULTS

rRNA gene restriction patterns. Analysis of rRNA genes with *Bgl*II produced four different restriction patterns, and the 93 strains of *V. cholerae* O139 could be differentiated into four different ribotypes (ribotypes I through IV) (Fig. 2), whereas all 24 isolates of El Tor vibrios belonged to a single ribotype (ribotype V) (Fig. 3). The rRNA gene restriction patterns were reproducible in repeated assays and consisted of 10 to 14 bands between 11 and 1.6 kb in size. Of the 65 strains of O139 vibrios isolated during 1992 and 1993 in Bangladesh and India, 28

isolates (43.1%) belonged to ribotype I, 37 isolates (56.9%) belonged to ribotype II, and the single CT-negative O139 strain from Argentina belonged to ribotype III. Of 27 O139 strains isolated during 1995 and 1996, 16 isolates (59.2%) belonged to ribotype IV and 11 isolates (40.7%) belonged to ribotype II (Table 1). The restriction pattern representing ribotype IV contained a unique band of 3.0 kb in size which was not present in any of the other restriction patterns (Fig. 2).

Restriction patterns of the *ctxA* gene. Restriction fragment length polymorphism (RFLP) analysis of the *ctxA* gene with the enzyme *Bgl*II revealed four different restriction patterns (A through D) for the O139 strains and two different patterns (E and F) for the El Tor strains. The *ctxA* patterns consisted of one to four bands between 12.2 and 2.7 kb (Fig. 4 and 5). The restriction endonuclease *Bgl*II does not have any recognition sequence within the *ctxA* gene (20), and consequently, the number of bands comprising each *ctxA* restriction pattern represented the possible number of copies of the *ctxA* gene carried by the strain. Three of the *ctxA* restriction patterns (A through C) were shared by O139 strains isolated from its initial outbreak during 1992 and 1993, whereas 16 of the 27 reemerged O139 strains isolated during 1995 and 1996 produced restriction pattern D. The remaining 11 strains produced restriction pattern C. Thus, 93 strains of *V. cholerae* O139 were differentiated into four different *ctx* genotypes based on the *Bgl*II restriction patterns of their *ctxA* genes (Table 1).

Analysis of the *rfb* gene cluster. PCR-based analysis of the O139 and El Tor vibrios with specific primers corresponding to six defined regions of the *rfb* gene cluster and flanking sequences of *V. cholerae* O1 (Table 2) showed that while all six regions could be amplified from the El Tor strains, the O139 strains showed positive amplification in only two of the six PCR assays. These two assays amplified a 497-bp region of the *rfaD* locus and a 394-bp region corresponding to the *rfbQRS* locus in all of the toxigenic O139 vibrios. The *rfbQRS* locus was also amplified from four of seven non-O1, non-O139 strains. However, the CT-negative strain of *V. cholerae* O139 from Argentina and all seven environmental isolates of non-O1, non-O139 vibrios from Bangladesh produced a slightly smaller-sized PCR product (~0.4 kb) corresponding to the *rfaD* locus (Fig. 6). All O139 strains as well as three of seven non-O1, non-O139 strains were negative in the PCR assays for chromosomal regions representing *rfbDEG*, *rfbNO*, *ompX*, *orf2*, and *orf3* of the

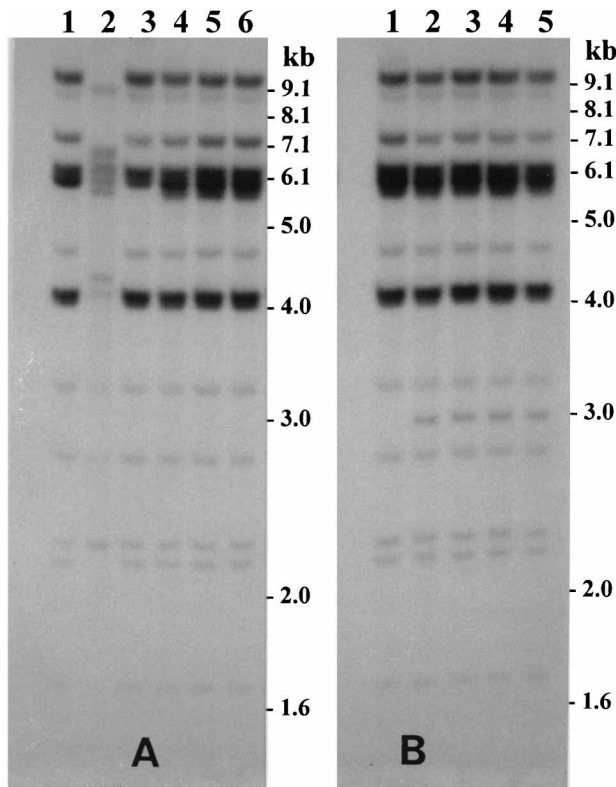


FIG. 2. Southern hybridization analysis of rRNA genes in *V. cholerae* O139 strains isolated during 1992 to 1993 (A) and during 1995 to 1996 (B). Genomic DNA was digested with *Bgl*I and probed with a 7.5-kb *Bam*HI fragment of the *E. coli* rRNA clone pKK3535. (A) Lanes 1 and 3 show *Bgl*I restriction pattern I, lanes 4 through 6 show restriction pattern II, and lane 2 shows restriction pattern III (produced by the CT-negative O139 strain from Argentina). (B) Lane 1 shows restriction pattern II and lanes 2 through 5 show restriction pattern IV (produced by the new clone of *V. cholerae* O139). Numbers indicating molecular sizes of bands correspond to bands of a 1-kb DNA ladder (Bethesda Research Laboratories) used as molecular size markers.

rfb gene cluster of *V. cholerae* O1 (Table 3). All PCR-generated amplicons hybridized with respective probes derived from strain 569B in hybridization assays. Southern blot hybridization with a probe for *V. cholerae* O139 O antigen-specific genes showed the presence of the O139-specific DNA in all O139 strains.

Analysis of the *tcp* and *toxR* genes. All El Tor and O139 strains except the CT-negative O139 strain from Argentina were positive for *tcpA*, *tcpI*, and the *toxR* genes. A PCR assay for *tcpA* amplified a 0.47-kb portion of the *tcpA* gene in all strains. This result was characteristic of the El Tor biotype *tcpA* gene. A PCR assay for the *tcpI* gene produced an amplicon of 2.1 kb from all toxigenic O139 and El Tor strains. Colony blot hybridization revealed that all of the toxigenic El Tor and O139 strains in this study carried the *toxR* gene.

DISCUSSION

The emergence of *V. cholerae* O139 received global attention due to its rapid spread as a new epidemic strain of cholera and was recorded as the first non-O1 *V. cholerae* capable of causing epidemic outbreaks (1, 29). Soon after the emergence of *V. cholerae* O139, the existing strains of *V. cholerae* O1 were almost completely displaced and the O139 vibrios continued to dominate until the emergence of a new clone of El Tor vibrios

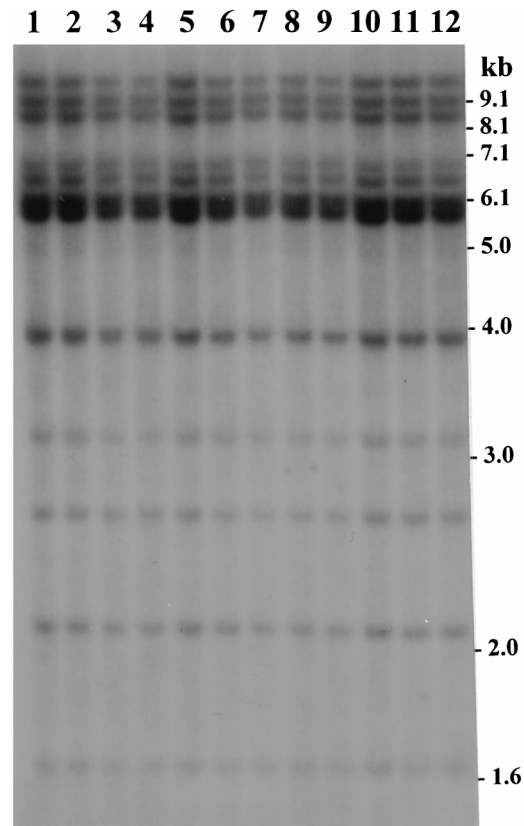


FIG. 3. Southern hybridization analysis of rRNA genes in *V. cholerae* O1 biotype El Tor isolated in Dhaka and five rural districts in Bangladesh during 1995 to 1996. Genomic DNA was digested with *Bgl*I and probed with the rRNA gene probe. All strains produced rRNA gene restriction patterns characteristic of the recently emerged clone of El Tor vibrios (ribotype V). Lane numbers and regions of isolation of the strains are as follows: lanes 1 and 2, Dhaka; lanes 3 and 4, Kurigram; lanes 5 and 6, Mymensingh; lanes 7 and 8, Sunamganj; lanes 9 and 10, Noakhali; and lanes 11 and 12, Jhalokati. Numbers indicating molecular sizes of bands correspond to bands of a 1-kb DNA ladder (Bethesda Research Laboratories).

which transiently displaced the O139 vibrios during 1994 and 1995 (6, 7, 32). However, the reemergence of *V. cholerae* O139 since June 1995 and its coexistence with the El Tor vibrios need to be explained. Previously recorded events of the appearance and disappearance of different clones of toxigenic *V. cholerae* in Bangladesh (1, 7, 10, 31–33) suggested that the persistence and domination by particular clones of toxigenic *V. cholerae* possibly occur through a competitive mechanism which may involve unidentified environmental factors, preexisting immunity in the host population, and perhaps antigenic characteristics of particular pathogenic clones. Our study was designed to investigate clonal diversity among O139 vibrios and ascertain whether the reemerged O139 strains represent a new clone of *V. cholerae* O139 which is able to compete better than the previously isolated O139 vibrios with the newly emerged clone of El Tor vibrios (7). We have previously examined restriction patterns of conserved rRNA genes (ribotypes) and CT genes (*ctxA*) or DNA flanking these genes to differentiate among clones of toxigenic *V. cholerae* which are otherwise phenotypically identical (7–10). These studies have demonstrated that the restriction patterns are reproducible and may be considered fairly stable markers for identifying different clones. In addition to analyzing RFLPs in genes for rRNA and in *ctxA*, for this study we performed a comparative

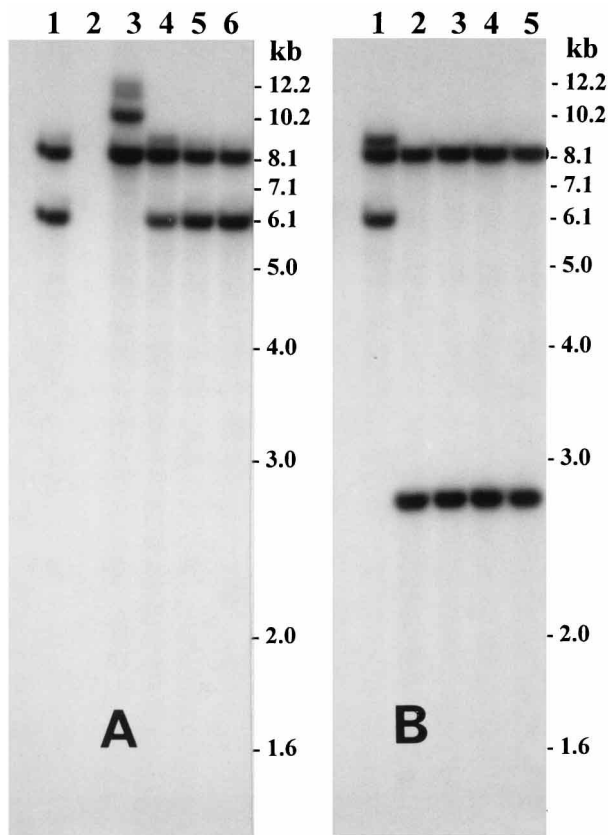


FIG. 4. Southern hybridization analysis of *ctxA* genes in *V. cholerae* O139 strains isolated during 1992 to 1993 (A) and during 1995 to 1996 (B). Genomic DNA was digested with *Bgl*I and probed with a 550-bp fragment of the A subunit of the CT gene. (A) Lanes 1, 5, and 6 show restriction pattern A, lane 3 shows restriction pattern B, and lane 4 shows restriction pattern C. Lane 2 contained genomic DNA from the CT-negative O139 strain isolated in Argentina. (B) Lane 1 shows restriction pattern A, whereas lanes 2 through 5 show restriction pattern D produced by the new clone of *V. cholerae* O139. Numbers indicating molecular sizes of bands correspond to bands of a 1-kb DNA ladder (Bethesda Research Laboratories) used as molecular size markers.

analysis of the chromosomal regions responsible for O antigen biosynthesis (*rfb* gene cluster) in the El Tor and O139 vibrios to study whether the genetic changes which led to the emergence of strains belonging to the O139 serogroup were similar in all strains of *V. cholerae* O139.

Clonal diversity of *V. cholerae* O139 strains. In a previous study by Popovic et al. (27), two different ribotypes were identified among *V. cholerae* O139 isolated from epidemics during 1992 and 1993 in India and Bangladesh. We also reported the rRNA gene restriction patterns of O139 vibrios isolated in Bangladesh and a CT-negative O139 strain isolated in Argentina, demonstrating that the latter isolate belonged to a ribotype widely different from the epidemic O139 vibrios (9, 10). In the present study, the 93 strains of *V. cholerae* O139 could be differentiated as belonging to four different ribotypes based on the *Bgl*I cleavage patterns of their rRNA genes (Fig. 2). The O139 vibrios isolated from epidemic outbreaks in 1992 to 1993 were of the two previously reported ribotypes (I and II), whereas 59.2% (16 of 27) of the reemerged O139 vibrios isolated during 1995 and 1996 belonged to a single new ribotype (IV). The restriction pattern representing ribotype IV produced by the reemerged O139 strains in this study have not been reported previously by us or other investigators who have

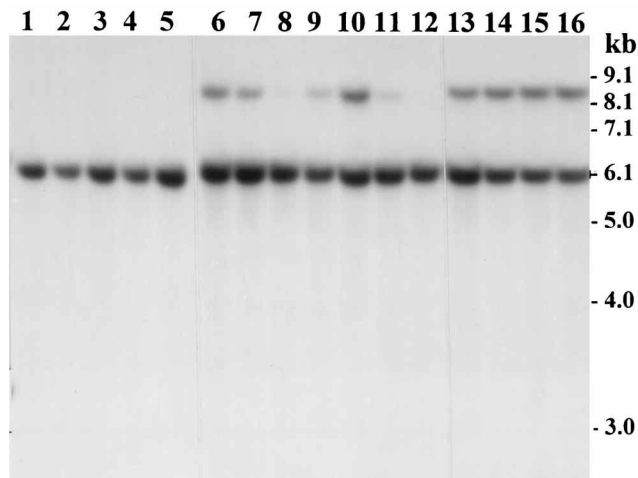


FIG. 5. Southern hybridization analysis of *ctxA* genes in *V. cholerae* O1 biotype El Tor isolated in Dhaka and five rural districts in Bangladesh during 1995 and 1996. Genomic DNA was digested with *Bgl*I and probed with the *ctxA* gene probe. Lanes 1 through 5, 8, and 12 show the restriction pattern corresponding to *ctxA* genotype E, whereas lanes 6, 7, 9, through 11 and 13 through 16 show the restriction pattern corresponding to *ctxA* genotype F. Lane numbers and regions of isolation of the strains are as follows: lanes 1 and 2, Jhalokati; lanes 3, 4, and 6, Dhaka and Noakhali; lane 5, Kurigram; lanes 7 and 8, Sunamganj; lanes 9 and 10, Mymensingh; and lanes 11 through 16, Noakhali. Numbers indicating molecular sizes of bands correspond to bands of a 1-kb DNA ladder (Bethesda Research Laboratories).

analyzed a large number of El Tor, classical, and O139 strains from different countries (9, 10, 17, 26, 27). This finding suggested that the O139 strains belonging to ribotype IV represent a new clone of *V. cholerae* O139.

Probing of the *Bgl*I restriction fragments of the chromosome for the *ctxA* gene also revealed differences among the O139 vibrios belonging to different ribotypes. In *V. cholerae*, the genes encoding CT (*ctxAB*) are part of a larger genetic element (CTX genetic element) consisting of at least five genes and

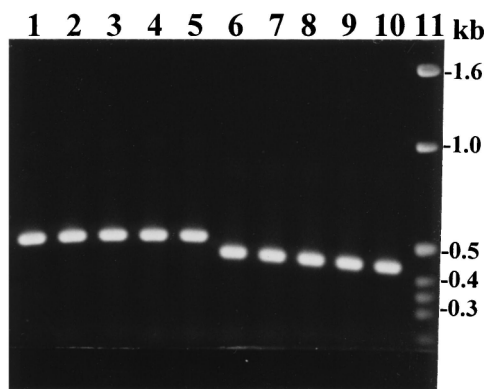


FIG. 6. Primers corresponding to the *rfaD* gene of *V. cholerae* O1 were used to amplify the corresponding genomic regions of *V. cholerae* O139 and non-O1, non-O139 strains by PCR. The PCR products were analyzed by agarose gel electrophoresis to verify the expected sizes of the amplicons. Lane 1 represents PCR amplicon derived from classical strain 569B, lanes 2 shows the amplification products from a *V. cholerae* O1 El Tor strain, lanes 3 through 5 show PCR products from *V. cholerae* O139 Bengal strains, lane 6 shows the PCR amplicon derived from the non-Bengal O139 strain isolated in Argentina, lanes 7 through 10 show the PCR products derived from four non-O1, non-O139 strains isolated from surface water in Bangladesh, and lane 11 shows low-molecular-weight fragments of a 1-kb DNA ladder (BRL) used as molecular size markers.

TABLE 3. Analysis of the *rfb* gene clusters and flanking regions of 91 *V. cholerae* O139, 24 *V. cholerae* O1, and 7 *V. cholerae* non-O1, non-O139 strains isolated between 1993 and 1996

Strain	Country	Yr(s) of isolation	No. of isolates studied	Presence of indicated region of the <i>V. cholerae</i> O1 <i>rfb</i> gene cluster or flanking region at nucleotides ^a :						Presence of O139-specific DNA ^a
				55-552 (<i>rfaD</i>)	4273-7072 (<i>rfbDEG</i>)	14031-14915 (<i>rfbNO</i>)	16373-16767 (<i>rfbQRS</i>)	18136-19298 (<i>ompX</i>)	19515-20101 (<i>orf2-orf3</i>)	
<i>V. cholerae</i> O139	Bangladesh	1993	56	+	-	-	+	-	-	+
	India	1992	7	+	-	-	+	-	-	+
	Argentina	1993	1	+ ^b	-	-	+	-	-	+
	Bangladesh	1995-1996	27	+	-	-	+	-	-	+
<i>V. cholerae</i> O1, biotype El Tor	Bangladesh	1995-1996	24	+	+	+	+	+	+	-
<i>V. cholerae</i> non-O1, non-O139	Bangladesh	1994-1995	2	+ ^b	-	-	+	+	+	-
	Bangladesh	1994-1995	2	+ ^b	-	-	+	-	-	-
	Bangladesh	1994-1995	3	+	-	-	-	-	-	-

^a Analysis of *rfb* genes was done by PCR or DNA hybridization assays as described in Materials and Methods.

^b Molecular sizes of these PCR products were smaller than the expected size (see the text).

flanked by two or more copies of a repeated sequence (25, 39). Although there is very little variation among the genes encoding CT in different strains, RFLP 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 this study, the O139 strains were grouped into four different *ctx* genotypes based on their *ctxA* restriction patterns (Table 1). While *ctx* genotypes A through C were shared by the O139 strains isolated after the initial emergence of O139 vibrios, 16 of the 27 isolates of the reemerged O139 strains, which belonged to the new ribotype (IV), belonged to a new *ctx* genotype (D). Hence, the ribotype data and the *ctx* genotype data agreed, providing further evidence that these reemerged *V. cholerae* O139 strains isolated in Bangladesh represent a new clone of the Bengal vibrios.

Origin of *V. cholerae* O139. Previous reports comparing *V. cholerae* O139 and *V. cholerae* O1 (4, 5, 9, 19, 27, 36, 40) by ribotyping and analysis of the *ctxA*, *tcpA*, and *toxR* genes and genes responsible for the biosynthesis of O1- and O139-specific O antigens (*rfb* genes) suggested that strains belonging to the O139 serogroup may have emerged from a genetic change in the serotype-specific genes of a toxigenic El Tor strain. These studies indicated that O139 strains resulted from the ancestral El Tor strain due to insertion of a large foreign genomic region encoding the O139-specific genes and simultaneous deletion of most of the O1-specific *rfb* gene cluster. The donor for the O139-specific DNA in this horizontal gene transfer event has, however, not been identified. In agreement with previous reports, a large region of chromosomal DNA representing the O1-specific *rfb* gene cluster, including regions representing *rfbDEG*, *rfbNO*, *ompX*, *orf2*, and *orf3*, were found to be absent in all O139 strains analyzed in this study. Regions corresponding to the *rfaD* gene of *V. cholerae* O1, located upstream of the *rfaA* gene, and those corresponding to *rfbQRS*, located upstream of *ompX*, were present in the O139 strains. Sequence corresponding to *rfbQRS* was, however, also present in the non-O1, non-O139 strains tested. In consideration of the arrangement of genes or open reading frames (ORFs) in the *rfb* gene cluster (19) and its flanking regions in *V. cholerae* O1, it seems likely that the *rfbQRS* region found in the O139 vibrios is part of the possible acquired DNA and not remnants of the *rfb* cluster of the parental O1 strain. This possibility is in agreement with results of a previous study (36) in which the region

corresponding to *rfbQRS* in O139 vibrios was demonstrated to constitute a single ORF closely resembling an insertion element, as opposed to three separate ORFs found in the O1 strains.

Possible mechanisms, including complex genetic recombination and rearrangements or simple bacteriophage-mediated transduction, have been proposed to explain the gene transfer event that gave rise to the O139 vibrios (36). In our study, PCR-based amplification with primers corresponding to the *rfaD* locus showed that the sizes of the amplicons in all toxigenic O139 strains and El Tor strains were similar but that the amplification product of the CT-negative O139 strain was smaller in size (Fig. 6) and was similar to those produced by several non-O1, non-O139 strains isolated from the environment (Table 3). The CT-negative O139 strain also lacked the *tcpA* gene, which encodes the major subunit of toxin-coregulated pili (TCP), as well as the *tcpI* and *toxR* genes, which regulate and fine tune the expression of TCP in *V. cholerae* O1 strains (19, 24, 38). This suggested that the CT-negative O139 strain possibly originated from a non-O1 strain but that the progenitor of the O139 Bengal strains may have been an El Tor strain. Thus, the serotype-specific genetic change which resulted in the emergence of the O139 serogroup occurred in more than one ancestral strain of *V. cholerae*. It is, however, not clear whether the conversion of a non-O1 strain to a non-Bengal O139 strain and that of an El Tor strain to the O139 Bengal strain were entirely independent events or whether a non-Bengal O139 strain had acted as the donor of the O139-specific DNA carried by the O139 Bengal strains. The existence of a second CT-negative non-Bengal O139 strain isolated in Sri Lanka (28) is interesting.

Clonal diversity among the toxigenic O139 Bengal strains evidenced by RFLP in conserved rRNA genes and *ctxA* genes may have resulted from mutations and genetic rearrangements in an existing clone of O139 vibrios or from independent emergence of these clones from different progenitor El Tor strains through similar genetic changes in the serotype-specific genes. If either of these alternatives was the mechanism of emergence, the possibility of the appearance of new clones of O139 vibrios in the future cannot be ruled out.

Coexistence of O139 and El Tor vibrios in Bangladesh. The emergence of a new clone of El Tor vibrios (ribotype V) occurred after the previously existing El Tor vibrios were dis-

placed by the O139 vibrios during 1993. In a previous study, we detected the new clone of El Tor vibrios in the capital of Bangladesh, Dhaka, and assumed that the same clone of El Tor vibrios might have replaced *V. cholerae* O139 in other parts of the country as well (7). In the present study, the 24 El Tor strains were isolated from six different regions of Bangladesh (Fig. 1 and Table 1) and all of the strains were found to belong to ribotype V. This confirmed our earlier speculation and demonstrated the spread of the new El Tor vibrios throughout Bangladesh. However, these strains were found to comprise the *ctxA* genotypes E and F carrying a single copy and two copies of the CTX genetic element, respectively, whereas in the previous study all ribotype V strains were found to carry a single copy of the CTX element. This change may have occurred by duplication of the CTX element (25) under unidentified selection pressure and/or competition for persistence with O139 vibrios. In at least four rural districts as well as in the capital Dhaka (Fig. 1), *V. cholerae* O139 reemerged after June 1995 and now coexists with El Tor vibrios. Two of these rural districts, Mymensingh and Naogaon, situated in the northern and northwestern regions of Bangladesh did not experience the O139 outbreak during 1992 to 1993, but *V. cholerae* O139 was isolated from these regions during 1996. All *V. cholerae* O139 strains isolated from these two regions belonged to the new clone (Table 1). In the southern district of Jhalokati, however, the O139 vibrios belonged to one of the initially detected clones (ribotype II). In the capital Dhaka, where there is an enormous influx of people from all of the rural districts, both the new and the old clones of O139 were detected, suggesting that the new clone of O139 vibrios is spreading towards the central region of the country. It is, therefore, important to monitor the presence of this new clone of O139 vibrios in other parts of the country as well as in the adjoining areas of India to better understand the origin and spread of strains belonging to the new clone. It may be of interest that a recent resurgence of *V. cholerae* O139 has also been reported from Calcutta (22).

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, 8, 12, 31). The recent surveillance by the ECPP in rural districts of Bangladesh has observed coexistence of El Tor and O139 vibrios causing cholera outbreaks due to either of the serogroups, and it is difficult to predict the future course of *V. cholerae* O1 and O139 infections in Bangladesh. The two serogroups may coexist for a while or one may completely replace the other through the process of natural selection. A somewhat similar coexistence of classical and El Tor biotypes of *V. cholerae* O1 was demonstrated in Bangladesh between 1982 and 1992, after the resurgence of classical vibrios as a predominant epidemic strain in 1982, nearly 10 years after it had apparently been replaced by El Tor vibrios (31, 33). In this study, all of the CT-positive strains of *V. cholerae* O1 or O139, including strains belonging to the new clone of O139 vibrios, carried the genes for TCP and ToxR, essential for pathogenesis (14). Most of these strains were isolated from cholera outbreaks and hence have potential to cause further rapidly spreading epidemics. It is, therefore, important to closely monitor the present situation of coexistence of O1 and O139 vibrios, especially the clones that have emerged recently in Bangladesh, by clinical and environmental surveillance, as well as genetic fingerprinting, to detect the spread of these strains in Bangladesh and neighboring countries and predict possible epidemic outbreaks of cholera.

ACKNOWLEDGMENTS

This research was funded by the United States Agency for International Development (USAID) under grant HRN-5986-A-00-6005-00 with the ICDDR,B. The ICDDR,B is supported by countries and agencies which share its concern for the health problems of developing countries. Current donors providing core support include the aid agencies of the governments of Australia, Bangladesh, Belgium, Canada, China, Denmark, Japan, Saudi Arabia, Sri Lanka, Sweden, Switzerland, Thailand, the United Kingdom, and the United States; international organizations, including the Arab Gulf Fund, the Asian Development Bank, the European Union, the United Nations Children's Fund (UNICEF), the United Nations Development Programme (UNDP), the United Nations Population Fund (UNFPA), and the World Health Organization (WHO); private foundations, including the Aga Khan Foundation, the Child Health Foundation, the Ford Foundation, the Population Council, the Rockefeller Foundation, and the Sasakawa Foundation; and private organizations, including American Express Bank, Bayer AG, CARE, Family Health International, Helen Keller International, the Johns Hopkins University, Macro International, the New England Medical Center, Procter Gamble, RAND Corporation, SANDOZ, the Swiss Red Cross, the University of Alabama at Birmingham, the University of Iowa, and others.

We thank John Mekalanos, Harvard Medical School, Boston, Mass., for the ToxR clone and the *V. cholerae* O139-specific probe and Manzurul Haque for secretarial assistance.

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