Molecular Characterization of a New Ribotype of *Vibrio cholerae* O139 Bengal Associated with an Outbreak of Cholera in Bangladesh

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Vibrio cholerae **O139 Bengal initially appeared in the southern coastal region of Bangladesh and spread northward, causing explosive epidemics during 1992 and 1993. The resurgence of** *V. cholerae* **O139 during 1995 after its transient displacement by a new clone of El Tor vibrios demonstrated rapid changes in the epidemiology of cholera in Bangladesh. A recent outbreak of cholera in two north-central districts of Bangladesh caused by** *V. cholerae* **O139 led us to analyze strains collected from the outbreak and compare them with** *V. cholerae* **O139 strains isolated from other regions of Bangladesh and neighboring India to investigate their origins. Analysis of restriction fragment length polymorphisms in genes for conserved rRNA (ribotype) revealed that the recently isolated** *V. cholerae* **O139 strains belonged to a new ribotype which was distinct from previously described ribotypes of toxigenic** *V. cholerae* **O139. All strains carried the genes for toxin-coregulated pili (***tcpA*** and ***tcpI***) and accessory colonization factor (***acfB***), the regulatory gene ***toxR***, and multiple copies of the lysogenic phage genome encoding cholera toxin (CTXΦ) and belonged to a previously described ***ctxA*** genotype. Comparative analysis of the ***rfb*** gene cluster by PCR revealed the absence of a large region of the O1-specific ***rfb*** operon downstream of the ***rfaD*** gene and the presence of an O139-specific genomic region in all O139 strains. Southern hybridization analysis of the O139-specific genomic region also produced identical restriction patterns in strains belonging to the new ribotype and those of previously described ribotypes. These results suggested that the new ribotype of Bengal vibrios possibly originated from an existing strain of** *V. cholerae* **O139 by genetic changes in the rRNA operons. In contrast to previously isolated O139 strains which mostly had resistance to trimethoprim, sulfamethoxazole, and streptomycin encoded by a transposon (SXT element), 68.6% of the toxigenic strains analyzed in the present study, including all strains belonging to the new ribotype, were susceptible to these antibiotics. Molecular analysis of the SXT element revealed possible deletion of a 3.6-kb region of the SXT element in strains which were susceptible to the antibiotics. Thus,** *V. cholerae* **O139 strains in Bangladesh are also undergoing considerable reassortments in genetic elements encoding antimicrobial resistance.**

Vibrio cholerae O139 Bengal emerged as a second etiologic agent of cholera in 1992 and caused explosive epidemics throughout Bangladesh, India, and neighboring countries (4, 25, 26). In Bangladesh, this new serogroup of epidemic *V. cholerae* was first detected in the southern coastal districts and offshore islands (29). The spread of the epidemic initially remained largely confined to the coastal districts, where the aquatic environment is saline and typical of the Bay of Bengal. Subsequently, *V. cholerae* O139 spread to the northeastern and north-central regions of the country and caused outbreaks of cholera (29). However, during 1994 and until 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 *V. cholerae* O139 continued to exist (7, 9, 29). 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. Analysis of these isolates revealed the emergence of a new clone of *V. cholerae* O139, and epidemiological assessment suggested that the new clone probably originated in the northern region of the country and spread toward the south (9). Recent surveillance by the Epidemic Control Preparedness Program of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), during November 1997 revealed an outbreak of cholera due to *V. cholerae* O139 in Mymensingh and Kishoreganj, two rural districts of Bangladesh situated north of Dhaka. A preliminary estimate revealed that over 50,000 cases of cholera and at least 34 deaths occurred during this outbreak (28a). Details of the surveillance will be published elsewhere. In the present study, we used molecular techniques to characterize *V. cholerae* O139 strains isolated from the recent outbreak and compared these with *V. cholerae* O139 strains isolated from other parts of Bangladesh and neighboring countries between 1995 and 1998 to study clonal relationships and understand the origins of these recently emerged epidemic strains.

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MATERIALS AND METHODS

V. cholerae **strains.** A total of 68 clinical isolates of *V. cholerae* O139 were included in this study. Strains isolated in Bangladesh consisted of 19 isolates from

TABLE 1. Analysis of genes for rRNA, CT (*ctxA*), and the SXT element in clinical strains of toxigenic *V. cholerae* O139 isolated between 1995 and 1998[a]

Yr of isolation	Place of isolation	No. of isolates	rRNA gene restriction pattern	*ctxA* genotype	SXT genotype	Antimicrobial resistance pattern[b]
1995	Matlab, Bangladesh	5	I	A	1	S^r SXT^r
1995	Matlab, Bangladesh	6	II	A	1	S^r SXT^r
1997	Matlab, Bangladesh	5	II	B	2	Susceptible
1996	Jhalokati, Bangladesh	2	II	B	2	Susceptible
1995	Dhaka, Bangladesh	5	II	A	1	S^r SXT^r
1996	Dhaka, Bangladesh	7	II	B	2	Susceptible
1996	Sunamganj, Bangladesh	2	II	B	1	S^r SXT^r
1997	Bakerganj, Bangladesh	1	II	B	2	Susceptible
1998	Bakerganj, Bangladesh	6	II	B	2	Susceptible
1997	Mymensingh, Bangladesh	8	III	B	2	Susceptible
1997	Kishoreganj, Bangladesh	11	III	B	2	Susceptible
1997	Bangkok, Thailand	3	II	B	1	S^r SXT^r
1997	Calcutta, India	6	II	A	2	Susceptible

[a] All strains were negative in PCR assays for chromosomal regions representing *rfbDEG*, *rfbNO*, *ompX*, *orf2*, and *orf3* of the *rfb* gene cluster of *V. cholerae* O1. Ribotypes, *ctxA* genotypes, and SXT genotypes are based on *Bgl*I restriction patterns of the respective genes and their flanking chromosomal sequences.

[b] Strains designated "Susceptible" were susceptible to tetracycline, ampicillin, chloramphenicol, gentamicin, ciprofloxacin, norfloxacin, nalidixic acid, streptomycin, and SXT. S^r, streptomycin resistant; SXT^r, SXT resistant.

the cholera outbreak in 1997 in two north-central districts of Bangladesh and 39 strains isolated in other regions of Bangladesh between 1995 and 1998. Other strains consisted of six strains isolated in India in 1997 (courtesy of G. B. Nair, National Institute of Cholera and Enteric Diseases, Calcutta), three strains isolated in Thailand (Armed Forces Research Institute of Medical Sciences, Bangkok), and a single nontoxigenic strain isolated in Argentina in 1993. Strains were stored either in a lyophilized form or in sealed deep nutrient agar at room temperature at the culture collection of the ICDDR,B. Before use, the identities of the cultures were confirmed by biochemical reaction and serology (36). Details of the strains are presented in Table 1.

Probes and hybridization. Colony blots were prepared with nylon filters (Hybond; Amersham International PLC, Aylesbury, United Kingdom) and processed by a standard method (16). 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 DNA was fixed to the nylon membrane by exposure to UV light for 3 min in accordance with the supplier's instructions. For preparation of DNA blots, total cellular DNA was isolated from overnight cultures as described previously (32). Five-microgram aliquots of DNA were digested with the appropriate restriction enzymes (Bethesda Research Laboratories [BRL], Gaithersburg, Md.), electrophoresed in 0.8% agarose gels, and blotted onto nylon membranes (Hybond; Amersham) by Southern blotting (30).

The gene probe used in this study to detect the CTX genetic element was a 0.5-kb *Eco*RI fragment of pCVD27 (12) containing part of the *ctxA* gene. The *toxR* gene probe was a 2.4-kb *Bam*HI fragment of pVM7 (18). The rRNA gene probe consisted of a 7.5-kb *Bam*HI fragment of the *Escherichia coli* rRNA clone pKK3535 (3, 32). The O139-specific DNA probe was a 1.3-kb *Eco*RI fragment of pCRII-A3 (21), and the SXT probe was a *Not*I fragment of pSXT1 (35). The probes were labeled by random priming (11) with a random primer DNA labeling kit (BRL) and [α-^{32}P]dCTP (3,000 Ci/mmol) (Amersham). Southern blots and colony blots were hybridized with the labeled probes, and autoradiographs were developed as described by us previously (7–9).

PCR assays. Presence of the toxin-coregulated pilus (TCP) pathogenicity island was determined by PCR assays specific for the *tcpA*, *tcpI*, and *acfB* genes. All oligonucleotides used either as probes or PCR primers were synthesized commercially by Oswel DNA Service (University of Edinburgh, Edinburgh, United Kingdom), and PCR reagents and kits were purchased from Perkin-Elmer Corporation (Norwalk, Conn.). Presence of *tcpA* genes specific for the classical and El Tor biotypes was determined by a multiplex PCR assay (14). The *tcpI* gene was detected by a specific PCR assay described by us previously (7). The *acfB* gene was detected by a PCR assay based on the published sequence of *acfB* (6) with two primers having the following sequences: 5'GGACCAAGCA TTATTATCTCT and 5'AATGATAAACTTACTGATTAA. The PCR assay amplified a 1.9-kb region of the *acfB* gene. Amplification was performed for 25 cycles consisting 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.

PCR assays for defined regions of the *rfb* gene cluster and adjoining sequences were performed with six different sets of primers derived from the sequence of the *V. cholerae* O1 *rfb* gene cluster, as described by us previously (9). The expected sizes of the amplicons were ascertained by electrophoresis in agarose gels, and the identity of each PCR product was also verified by Southern blot hybridization. PCR-negative strains were further confirmed for the absence of

the relevant genes by colony blot hybridization with the corresponding PCR-generated amplicons from a positive control strain, 569B, as specific probes.

Antimicrobial resistance. All *V. cholerae* isolates were tested for antimicrobial resistance by the method of Bauer et al. (2) with standard antibiotic disks (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) at the following antibiotic concentrations (in micrograms per disk): ampicillin, 10; chloramphenicol, 30; streptomycin, 10; tetracycline, 30; trimethoprim-sulfamethoxazole (SXT), 1.25 and 23.75, respectively; kanamycin, 30; gentamicin, 10; ciprofloxacin, 5; norfloxacin, 10; nalidixic acid, 30.

RESULTS AND DISCUSSION

The epidemiology of cholera in the flood plains of the northern region of Bangladesh had previously been shown to differ considerably from that in the southern coastal region (29), probably due to differences in the water ecologies between the two regions. 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 (7). The new clone of El Tor vibrios displaced the O139 vibrios during 1994 and 1995 in the central and northern areas of Bangladesh, but the O139 vibrios continued to exist and became endemic in the southern coastal region, where this serogroup was initially detected in 1993 (4, 29). During 1995 and 1996, after the reemergence of *V. cholerae* O139, molecular epidemiological studies suggested that strains isolated from the southern regions were remnants of the initial clones whereas those isolated in the northern districts belonged to a new clone (9). The present study was designed to ascertain whether strains isolated from the recent outbreak in the two north-central districts of Bangladesh also represented a new clone of *V. cholerae* O139.

Ribotype analysis. We have previously examined restriction patterns of conserved rRNA genes (ribotypes) and cholera toxin genes or chromosomal DNA flanking these genes to differentiate among clones of toxigenic *V. cholerae* which were otherwise phenotypically identical (7–9). These studies have also shown that *Bgl*I was the most discriminatory restriction enzyme used for ribotyping. In the present study, analysis of rRNA genes with *Bgl*I revealed three different restriction patterns (patterns I through III) among the toxigenic strains (Fig. 1). The nontoxigenic O139 strain from Argentina produced a restriction pattern which was widely different from those of the
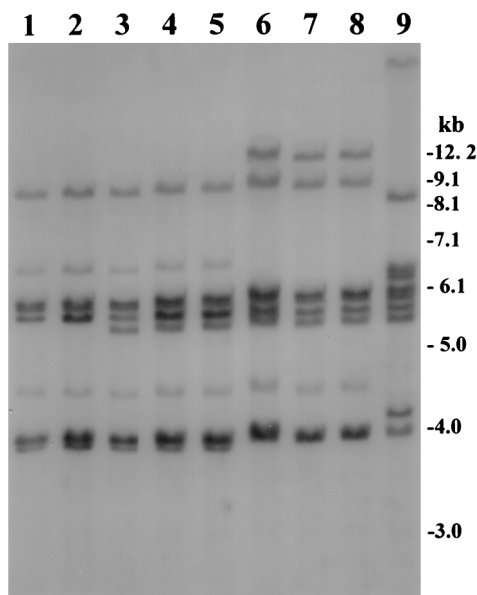
FIG. 1. Southern hybridization analysis of rRNA genes in *V. cholerae* O139 strains isolated between 1995 and 1998 in Bangladesh, India, and Thailand. Genomic DNA was digested with *Bgl*I and probed with a 7.5-kb *Bam*HI fragment of the *E. coli* rRNA clone pKK3535. Restriction pattern I, produced by two strains belonging to designated ribotype B-I isolated from Bangladesh and Thailand, respectively, are shown in lanes 1 and 2; restriction pattern II, produced by three strains belonging to ribotype B-II isolated in Bangladesh, India, and Thailand, respectively, are shown in lanes 3 through 5; and lanes 6 through 8 show restriction pattern III, produced by the new ribotype of *V. cholerae* O139 designated B-III, isolated from a recent outbreak in two north-central districts of Bangladesh. The pattern produced by the nontoxigenic O139 strain isolated in Argentina is shown in lane 9. Numbers indicating molecular sizes of bands correspond to a 1-kb DNA ladder (BRL) used as a molecular size marker.

toxigenic strains described by us previously (9). All of the 19 strains isolated from the recent outbreak produced restriction pattern III (Fig. 1), which was different from the previously reported ribotype patterns of *V. cholerae* isolates belonging to the O139 or O1 serogroup (7–9, 23, 24). Strains isolated in India and Thailand analyzed in the present study produced either restriction pattern I or II (Table 1). Thus, *V. cholerae* O139 strains isolated from the recent epidemic in the north-central region of Bangladesh belonged to a new ribotype. The rRNA gene restriction patterns were reproducible in repeated assays and consisted of seven to eight major bands of between 12.5 and 3.9 kb in size. The restriction pattern representing the new ribotype contained a unique band of 12.5 kb which was not present in any of the other restriction patterns (Fig. 1). Hence, the O139 strains isolated from the recent epidemic were distinguishable from previously described O139 vibrios based on the ribotype pattern. The other two patterns detected among toxigenic O139 strains in the present study (patterns I and II) have been described previously (9), and these strains are likely to be remnants of the original clones of *V. cholerae* O139 which emerged during 1992 and 1993. However, another clone of toxigenic *V. cholerae* (9) which appeared transiently in 1996 was not found among the isolates analyzed in the present study. The detection of strains belonging to a new ribotype in the present study suggested that *V. cholerae* O139 strains are undergoing genetic changes leading to increased ribotype diversity. At present there is no standardized systematic nomenclature for designating ribotypes of *V. cholerae* O139 Bengal; hence, strains belonging to similar ribotypes have been designated differently in previous reports (8, 9, 24). With the pros-

pect of emerging ribotype diversity among *V. cholerae* O139 isolates, it is important to establish a systematic nomenclature for identifying and monitoring the emergence and spread of epidemic O139 strains. We propose that the *V. cholerae* O139 Bengal strains producing the three different ribotype patterns, I through III (Fig. 1), be designated ribotypes B-I, B-II, and B-III (Table 1). Since previous studies have suggested that the nontoxigenic non-Bengal O139 strain (9) isolated in Argentina has an origin widely different from the epidemic Bengal strains, we propose the ribotype designation NB-1 for this strain. Analysis of a large number of *V. cholerae* O139 strains isolated in different countries is being carried out in our laboratory to further develop a standardized system for ribotype designations of toxigenic *V. cholerae*.

Analysis of virulence genes. Previous studies have demonstrated that the rRNA gene restriction patterns (ribotypes) could be considered fairly stable markers for identifying different clones, and generally different ribotypes have also shown differences in the restriction patterns of several other genes studied (7, 9). We subjected the strains belonging to the new ribotype to further analysis to determine whether major pathogenic genes carried by strains of the new ribotype showed genetic dissimilarities with strains of the other ribotypes studied. The major colonization factor of toxigenic *V. cholerae* is TCP, the expression of which is under genetic control of the transcriptional activator ToxR and is coregulated with the expression of cholera toxin (CT) (18, 33). Although the major subunit of TCP is encoded by the *tcpA* gene, the formation and function of the pilus assembly requires the products of a number of other genes located on a large DNA region referred to as the TCP pathogenicity island, which includes the *tcp* and *acf* gene clusters (13, 15). Analysis of genes for the TCP pathogenicity island with PCR assays for the *tcpA*, *tcpI*, and *acfB* genes showed that all strains carried the TCP pathogenicity island. The PCR assay for *tcpA* amplified a 0.47-kb portion of the *tcpA* gene in all strains. The PCR assays for the *tcpI* and *acfB* genes produced amplicons of 2.1 and 1.9 kb, respectively, from all strains tested. Colony blot hybridization revealed that all strains in the present study also carried the *toxR* gene. Like previously described O139 Bengal vibrios, the *tcpA* amplicon derived from strains belonging to the new ribotype of *V. cholerae* O139 was identical to that produced by El Tor strains of *V. cholerae* (9). The *ctxAB* operon, which encodes the A and B subunits of CT, is part of a larger genetic element originally termed the CTX genetic element (22). Recent studies have shown that the CTX genetic element corresponds to the genome of CTXΦ, a lysogenic filamentous bacteriophage (34). In the present study, restriction fragment length polymorphism analysis of the *ctxA* gene and its flanking chromosomal sequence with the enzyme *Bgl*I revealed two different restriction patterns (A and B) for O139 strains. The *ctxA* restriction patterns consisted of either two or three bands between 9.1 and 6.1 kb (Fig. 2). Since the *ctxA* gene is known to have no internal *Bgl*I site (17), the number of bands represented the approximate number of copies of the integrated CTXΦ genome. Both of the *ctxA* restriction patterns produced by strains analyzed in the present study have been reported previously for *V. cholerae* O139 strains (9). All strains collected from the recent outbreak produced pattern B (Fig. 2) and carried three copies of the CTXΦ genome. We have previously proposed that origination of new toxigenic strains of *V. cholerae* is associated with the induction and propagation of CTXΦ (10). Diversification of the *ctxA* genotype among different strains may be a result of integration of CTXΦ into different regions of the host chromosome specified by the presence of a 17-bp attachment sequence referred to as *att*RS (34). Hence the new ribotype of *V.*
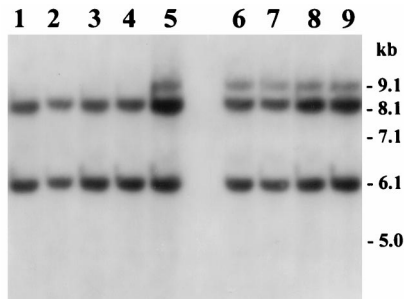
FIG. 2. Southern hybridization analysis of *ctxA* genes in *V. cholerae* O139 strains isolated from the epidemic in the north-central region of Bangladesh (lanes 6 through 9) in 1997 and from other districts between 1995 and 1998 (lanes 1 through 5). Genomic DNA was digested with *Bgl*I and probed with a 0.5-kb fragment of the *ctxA* gene. Restriction patterns corresponding to *ctxA* genotype A are shown in lanes 1 through 4, whereas those representing *ctxA* genotype B are shown in lanes 5 through 9. Numbers indicating molecular sizes of bands correspond to a 1-kb DNA ladder (BRL) used as a molecular size marker.
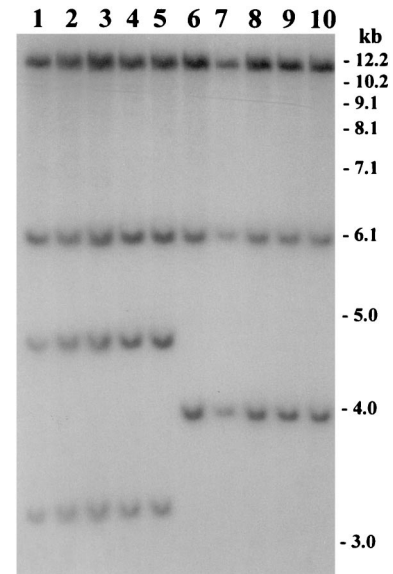

FIG. 3. Analysis of the SXT element in *V. cholerae* O139 strains isolated between 1995 and 1998 in Bangladesh. Genomic DNA was digested with *Bgl*I and probed with the SXT gene probe. Lanes 1 through 5 show restriction patterns corresponding to SXT genotype 1, whereas lanes 6 through 10 represent SXT genotype 2. Lane numbers, place, and year of isolation of strains are as follows: 1 and 2, Matlab, 1995; 3 and 4, Dhaka, 1995; 5, Sunamganj, 1996; 6 and 7, Mymensingh, 1997; 8 and 9, Kishoreganj, 1997; 10, Bakerganj, 1998. Numbers indicating molecular sizes of bands correspond to a 1-kb DNA ladder (BRL).

cholerae O139 carries the *att*RS sequence at chromosomal sites similar to other previously described O139 vibrios. These results suggested that unlike the non-Bengal O139 strain from Argentina, which has been shown to have a non-O1 origin (9), the new ribotype of *V. cholerae* O139 is closely related other *V. cholerae* O139 Bengal vibrios.

Antimicrobial resistance. The O139 serogroup of *V. cholerae* which emerged during 1992 and 1993 was sensitive to tetracycline and showed a trend of increased resistance to SXT and streptomycin. Waldor and coworkers (35) reported the presence of a 62-kb self-transmissible transposon-like element (SXT element) encoding resistance to sulfamethoxazole, trimethoprim, and streptomycin in *V. cholerae* O139 strains isolated from this epidemic. The SXT element could be conjugally transferred from *V. cholerae* O139 to *V. cholerae* O1 and *E. coli* strains, where it integrated into recipient chromosomes in a site-specific *recA*-independent manner (35). In the present study, all strains of the new ribotype and 27 of 48 strains (56.25%) belonging to previously reported ribotypes were found to be sensitive to SXT and streptomycin (Table 1). All strains analyzed in the present study were also sensitive to tetracycline, ampicillin, chloramphenicol, gentamicin, ciprofloxacin, norfloxacin, and nalidixic acid. In keeping with the observations in Bangladesh, comparison of antibiotic resistance patterns between the O139 strains isolated during 1992 and 1993 and those isolated in 1996 and 1997 in India also showed that the latter strains were susceptible to SXT, unlike the O139 strains from 1992 and 1993 (19). To identify the genetic changes associated with the observed SXT sensitivity, we used a cloned SXT gene probe to study restriction fragment length polymorphisms in the SXT transposon. Two different *Bgl*I restriction patterns (patterns 1 and 2) of the SXT element were observed among the toxigenic O139 strains tested (Fig. 3). Strains producing pattern 2 were sensitive to SXT and streptomycin, whereas those producing pattern 1 were resistant to all three antibiotics. Further analysis of the restriction patterns suggested that the restriction site heterogeneity possibly occurred as a result of a deletion of an approximately 3.6-kb region of the SXT element in strains which were sensitive to SXT and streptomycin. Recent studies in India have also shown that O139 strains are becoming increasingly resistant to ampicillin and neomycin but increasingly susceptible to chloramphenicol and streptomycin (20). Considering the rapidly changing pattern of antibiotic resistance observed among *V. cholerae* O139, it appears that there is substantial mobility in

other genetic elements encoding antimicrobial resistance in *V. cholerae* O139.

Origin of the new ribotype of *V. cholerae* O139. Previous reports on the comparative analysis of *V. cholerae* O139 and *V. cholerae* O1 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 progenitor El Tor strain due to insertion of a large foreign genomic region encoding O139-specific genes and simultaneous deletion of most of the O1-specific *rfb* gene cluster (5, 31). The donor for the O139-specific DNA in this horizontal gene transfer event, however, has 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*, was found to be absent in all O139 strains analyzed in the present study. PCR-based comparative analysis of the O139 vibrios with specific primers corresponding to six defined regions of the *rfb* gene cluster and flanking sequences of *V. cholerae* O1 showed that while all six regions could be amplified from control El Tor strains, the O139 strains showed positive amplification in 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. All O139 strains were negative in the PCR assays for chromosomal regions representing *rfbDEG*, *rfbNO*, *ompX*, *orf2*, and *orf3* of the *rfb* gene cluster of *V. cholerae* O1. Southern hybridization analysis of genomic DNA with the O139-specific probe also produced identical restriction patterns for all O139 vibrios examined (data not shown). In addition, strains of the new ribotype belonged to a *ctxA* genotype which was identical to that of previously described O139 strains. Contrary to this, however, the recently reemerged O139 strains in India have been re-

ported to show an altered organization of the CTX element (28).

The present study demonstrates the emergence of strains belonging to a new ribotype which are otherwise identical to previously described clones of O139 vibrios. Considering the *ctxA* genotypes, the organization of the *rfb* genes, and the presence of other virulence genes tested, it appears that the new ribotype probably did not emerge from a recent gene transfer event comprising horizontal transfer of O139-specific genes from an unidentified donor; rather, it seems more likely that the new ribotype originated from an existing O139 strain. This agrees with a previous study by Ruiting and Reeves (27) suggesting that recombination between rRNA operons can give rise to ribotype diversity.

Epidemiological significance of ribotype diversity. Previously recorded events of appearance of new ribotypes of toxigenic *V. cholerae* in Bangladesh showed that the new ribotypes were also distinct in their *ctxA* genotypes, suggesting that the new ribotypes possibly emerged from nontoxigenic progenitors (7, 9). This would allow more phenotypic and genetic diversity among toxigenic *V. cholerae* strains. The present study, on the other hand, shows that strains belonging to the new ribotype are otherwise genetically similar to previously described O139 strains. Hence, strains belonging to the new ribotype have epidemic or pandemic potential similar to previously described O139 vibrios. However, the new ribotype pattern of the strains has epidemiological application as a marker in monitoring the spread of these pathogenic strains. In the present study, all 19 strains isolated from the recent cholera outbreak belonged to the new ribotype. This suggests that the recent outbreak in northern Bangladesh probably started from a point source and coincided with the origination of this new ribotype. Analysis of strains isolated from other areas of Bangladesh during the same period showed that these strains belong to previously described ribotypes, and hence the strains associated with the outbreak were largely confined to the two north-central districts of the country. During 1996, a new ribotype of *V. cholerae* O139 was detected in Bangladesh and in the same geographical region (9). However, in the present study we did not find the presence of any strain belonging to this ribotype. This suggests that in this region of Bangladesh the O139 vibrios are undergoing rapid genetic reassortment, resulting in transient appearance of different clones, whereas in the southern coastal region the original ribotypes still exist as endemic strains. The reasons for the rapid and transient emergence of different ribotypes of O139 vibrios in the northern region of Bangladesh are not clear.

Interplay of a variety of factors in the aquatic environment and genetic and phenotypic changes in *V. cholerae*, as well as the immune status of the human host, may contribute to the existence and dominance of different clones of toxigenic *V. cholerae*. We have previously reported different phenotypic and genetic changes in *V. cholerae* O139 in Bangladesh and the prevalence of different clones (1, 8). Although in the present study we did not detect any change in the major pathogenic genes carried by the strains belonging to the new ribotype, we did not rule out the possibility of simultaneous genetic changes occurring in the rRNA operons and other unidentified genes that might influence the prevalence of the O139 strains by interacting with environmental factors. In view of the fluctuation observed in the prevalence of *V. cholerae* O139 relative to that of *V. cholerae* O1 in human infection (7, 9) and rapid genetic and phenotypic changes, including changing patterns of antibiotic resistance, further studies are required to explain the appearance and disappearance of cholera epidemics and the mobility of genetic elements encoding virulence properties as well as antimicrobial resistance.

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