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Molecular Epidemiology of Toxigenic *Vibrio cholerae* in Bangladesh Studied by Numerical Analysis of rRNA Gene Restriction Patterns

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Cholera is endemic in Bangladesh, and a regular seasonal pattern of cholera epidemics occurs. We examined the clonal relationships among 103 clinical and environmental *Vibrio cholerae* isolates belonging to O1, O139, or non-O1 non-O139 serogroups isolated during epidemic and interepidemic periods in Bangladesh and compared them with those of 51 *V. cholerae* isolates from four countries in Asia and Africa. These studies were done by a computer-assisted numerical analysis of the restriction endonuclease cleavage patterns of rRNA genes (ribotypes). Unweighed pair-group cluster analysis of *Bgl*I- and *Hin*dIII-generated band patterns revealed 16 clusters. Ribotypes were defined as clusters of strains possessing >98% similarity. The results showed that 154 isolates could be differentiated into 15 different ribotypes, and strains belonging to 3 of these ribotypes (ribotypes I, V, and VIIIA and VIIIB) were isolated more frequently during the epidemic periods than during interepidemic periods in Bangladesh. Classical vibrios belonged to six different ribotypes (ribotypes I to VI), with a mean similarity coefficient of 0.84, and the El Tor vibrios belonged to five different ribotypes (ribotypes VIIIA and IX to XII), with a mean similarity coefficient of 0.82. A single clone of El Tor vibrios (ribotype XII) was resident in Tanzania, whereas Nigeria, Syria, and India shared toxigenic El Tor strains with Bangladesh. Cholera toxin (CT)-positive O139 vibrios isolated from Bangladesh and India belonged to a single ribotype (ribotype VIIIB) and were >98% similar to one of the ribotypes of El Tor vibrios (ribotype VIIIA), but a CT-negative O139 vibrio from Argentina (ribotype XIII) was <75% similar to the same cluster of El Tor vibrios, thus suggesting more than one possible origin for O139 vibrios. Strains belonging to the same ribotypes (ribotypes VIII to X) were isolated from both patients and surface water in Bangladesh, indicating possible transmission through surface water. A clone of a CT-positive environmental isolate of non-O1 *V. cholerae* (ribotype VII) was found to be closely related (76.3% similarity) to a clone of classical vibrios (ribotype I) and was only between 27.2 and 56.1% similar to clusters of El Tor, O139, and two other non-O1 nontoxigenic clones.

Epidemics of cholera caused by toxigenic *Vibrio cholerae* O1 and O139 represent a major public health problem in developing countries including Bangladesh (1, 13, 14, 22, 27). Cholera is endemic in southern Asia and parts of Africa and Latin America, where seasonal outbreaks occur. These outbreaks are particularly associated with poverty and poor sanitation (2). In Bangladesh, the number of cholera cases varies from year to year, but cholera shows a pronounced seasonality, with a high frequency of infection just after the monsoon season, i.e., from October to January inclusive (1, 24). During the past 30 years various changes in the properties of epidemic *V. cholerae* strains in Bangladesh have been noticed. These include the appearance and disappearance of multiple-drug-resistant strains (10, 19), the replacement of classical vibrios by El Tor vibrios, and a sudden reappearance of classical vibrios after 10 years (25, 26); most recently, cholera epidemics have been caused by a non-O1 *V. cholerae* strain (*V. cholerae* O139) (1, 22).

Epidemiological surveillance of cholera and comparative analysis of clonal relationships among strains collected during epidemic and interepidemic periods are essential for identifying potential epidemic strains and studying the evolution and spread of epidemic strains. Since the identification of such strains on the basis of phenotypic characters alone has proven to be unsatisfactory (12, 18, 32) (mainly because phenotypes may not be stably expressed and/or not sufficiently discriminatory for strain differentiation), analysis of restriction fragment length polymorphisms in one or more genes is becoming increasingly useful as a tool for typing toxigenic *V. cholerae* strains in epidemiological investigations (4, 15, 32, 35). A recent typing method involves the use of *Escherichia coli* rRNA gene probes to study the restriction fragment length polymorphisms of conserved rRNA genes (ribotyping) in different strains and has been useful in typing pathogenic bacteria (8, 30). Ribotyping has recently been used to study the molecular epidemiology of cholera in several countries (5, 6, 20, 21) on a limited scale. However, those studies involved manual comparison of ribosomal DNA (rDNA) band patterns derived from different strains and hence lacked a quantitative assessment of genetic similarities and differences among *V. cholerae* strains. The present study examined the molecular epidemiology of cholera in Bangladesh. The study was based on a computer-assisted numerical analysis of rRNA gene restriction patterns in *V. cholerae* strains isolated from patients and surface water samples during epidemic and interepidemic periods. These patterns were compared with those of *V. cholerae* strains iso-

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TABLE 1. rRNA gene restriction patterns and ribotypes of clinical and environmental isolates of *V. cholerae* obtained from Bangladesh and other countries

Strain	No. of strains	Country	Source	Yr of isolation	Presence of		rRNA gene restriction pattern with:		Designated ribotype
					ctx	*zot*	*Bgl*I	*Hin*dIII	
V. cholerae O1									
Classical[a]	10	Bangladesh	Patient	1961–1968	+	+	A	A	I
Classical	6	Bangladesh	Patient	1963	+	+	D	A	II
Classical	1	Bangladesh	Patient	1965	+	+	B	A	VI
Classical[a]	4	Bangladesh	Patient	1965	+	+	C	A	III
Classical	1	Bangladesh	Patient	1966	+	+	E	A	IV
Classical	4	Bangladesh	Patient	1982–1988	+	+	A	A	I
Classical[a]	5	Bangladesh	Patient	1989	+	+	F	B	V
El Tor[a]	12	Bangladesh	Patient	1969–1976	+	+	O	A	VIIIA
El Tor	2	Bangladesh	Patient	1990	+	+	K	A	IX
El Tor	3	Bangladesh	Patient	1991	+	+	L	A	X
El Tor[a]	3	Bangladesh	Patient	1991	+	+	O	A	VIIIA
El Tor	6	Bangladesh	Patient	1990–1992	+	+	N	A	XI
El Tor	14	Tanzania	Patient	1992	+	+	M	C	XII
El Tor	11	Nigeria	Patient	1992	+	+	O	A	VIIIA
El Tor	8	Syria	Patient	1992	+	+	K	A	IX
El Tor	1	Syria	Patient	1992	+	+	O	A	VIIIA
El Tor	6	India	Patient	1992	+	+	O	A	VIIIA
El Tor	3	India	Patient	1992	+	+	K	A	IX
El Tor	2	India	Patient	1992	+	+	L	A	X
El Tor[a]	7	Bangladesh	S. water[b]	1991–1993	+	+	O	A	VIIIA
El Tor	1	Bangladesh	S. water	1992	+	+	L	A	X
El Tor	4	Bangladesh	S. water	1994	+	+	K	A	IX
V. cholerae non-O1									
O139[a]	15	Bangladesh	Patient	1993	+	+	P	A	VIIIB
O139[a]	5	India	Patient	1993	+	+	P	A	VIIIB
O139	1	Argentina	Patient	1993	−	−	H	A	XIII
O139[a]	2	Bangladesh	S. water	1993	+	+	P	A	VIIIB
O139	2	Bangladesh	S. water	1994	+	+	P	A	VIIIB
Non-O139	2	Bangladesh	S. water	1986	−	−	I	A	XIV
Non-O139	3	Bangladesh	S. water	1987	+	+	G	D	VII
Non-O139	2	Bangladesh	S. water	1994	+	+	G	D	VII
Non-O139	5	Bangladesh	S. water	1994	−	−	I	A	XIV
Non-O139	3	Bangladesh	S. water	1993	−	−	J	A	XV

[a] Strains isolated during epidemic periods.
[b] S. water, surface water.

lated from four other countries in Asia and Africa to assess possible clonal relationships among the isolates.

MATERIALS AND METHODS

***V. cholerae* isolates.** A total of 154 *V. cholerae* isolates from six different countries consisting of strains belonging to O1, O139, or non-O1 non-O139 serogroups obtained either from patients or from surface water samples were included in the study. These isolates were stored either in lyophilized form or in sealed deep nutrient agar at room temperature at the culture collection of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). Clinical isolates from Bangladesh were obtained from patients who attended the treatment center of ICDDR,B, located in Dhaka, between 1961 and 1994. The environmental isolates were from surface water samples in Dhaka isolated between 1987 and 1994. Other Asian isolates consisted of 9 El Tor strains from Syria (courtesy of F. Harb, Public Health Laboratory, Damascus) and 11 El Tor strains and 5 O139 strains from India (courtesy of G. B. Nair, National Institute for Cholera and Enteric Diseases, Calcutta). The African strains consisted of 14 El Tor strains from Tanzania (courtesy of F. Mahlu, Muhimbili Medical Centre, Dar-es-Salam) and 11 El Tor strains from Nigeria (courtesy of H. van Vliet, World Health Organization, Lagos). A cholera toxin (CT)-negative O139 strain from a patient in Argentina was also included in the study. Before use the identity of the cultures was confirmed by biochemical reaction and serology (34), and the presence of cholera toxin (*ctx*) and zonula occludens toxin (*zot*) genes was determined by colony blot hybridization with gene probes as described previously (7). Details of the isolates are presented in Table 1.

Determination of rRNA gene restriction patterns. Southern blot hybridization with an rRNA gene probe was performed on all 154 *V. cholerae* isolates as we described previously (5). Briefly, high-molecular-weight chromosomal DNAs from these isolates were prepared (30), digested with the restriction enzyme *Bgl*I or *Hin*dIII (Bethesda Research Laboratories, Gaithersburg, Md.), electrophoresed in 0.8% agarose gels, and transferred onto nylon membranes (Hybond; Amersham International plc, Aylesbury, United Kingdom) by Southern blotting (29).

The probe DNA was a 7.5-kb *Bam*HI fragment of pKK3535 (3), which is a pBR322-derived plasmid containing an *E. coli* rRNA operon consisting of one copy each of the genes coding for 5S rRNA, 16S rRNA, 23S rRNA, and $tRNA^{Glu}$. The probe DNA was labeled by random priming (9) with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham) and a random primer DNA labeling system (Bethesda Research Laboratories).

Southern blots were prehybridized at 68°C for 2 h in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.15 M sodium citrate), 5× Denhardt solution (1× Denhardt solution is 0.02% polyvinylpyrrolidone, 0.02% Ficoll 400, and 0.02% bovine serum albumin), 0.5% sodium dodecyl sulfate (SDS), and 100 μg of freshly denatured sheared salmon sperm DNA per ml. The blots were then hybridized with freshly denatured labeled probe at 68°C for 18 h in 6× SSC–0.5% SDS–100 μg of salmon sperm DNA per ml. Hybridized blots were washed once in 2× SSC for 5 min at room temperature, two times in 2× SSC–0.1% SDS for 10 min at 65°C, and once in 0.2× SSC–0.1% SDS for 15 min at 65°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 (5). In order to clearly visualize both the faint and the dark bands, two autoradiographs were developed from each hybridized filter by exposing the films once for 24 h and then for 48 h with intensifying screens.

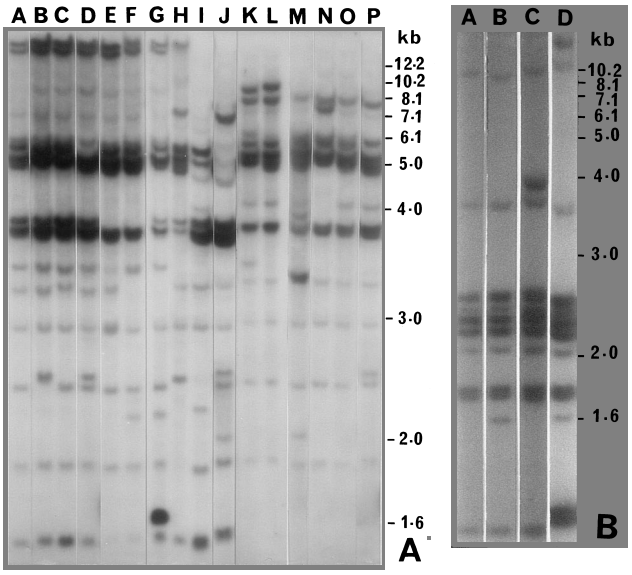

FIG. 1. Southern hybridization analysis of genomic DNAs from *V. cholerae* isolates digested with *Bgl*I (A) and *Hin*dIII (B) and probed with a 7.5-kb *Bam*HI fragment of the *E. coli* rRNA clone pKK3535. (A) Lanes A through P, *Bgl*I restriction patterns A through P of rRNA genes, respectively; (B) lanes A through D, *Hin*dIII restriction patterns A through D of rRNA genes, respectively. Numbers indicate the molecular sizes of the bands; a 1-kb DNA ladder (Bethesda Research Laboratories) was used as a molecular size marker.

Numerical analysis of rDNA bands. Banding profiles from *Bgl*I- and *Hin*dIII-digests were combined, and the total number of loci where bands occurred were counted. The absence or presence and intensities of bands at the loci were scored 0 or 1 to 3 for each strain. Computer-assisted analysis of the scores was performed with PhP-2 software (version 2; Biosys Inova; Luntmakargatan, Stockholm, Sweden) to calculate the similarity between each pair of isolates, expressed as a correlation coefficient, and to yield a similarity matrix consisting of $n \times (n - 1)/2$ correlation coefficients, where n is the number of isolates. The similarity matrix was clustered according to the unweighted pair-group method with arithmetic averages to produce a dendrogram (28). To enable high discrimination levels, clusters were defined as groups of isolates with >98% similarity. Clusters were designated ribotypes, and groups within a cluster that had greater than 98% similarity but that were not identical were further designated subribotypes.

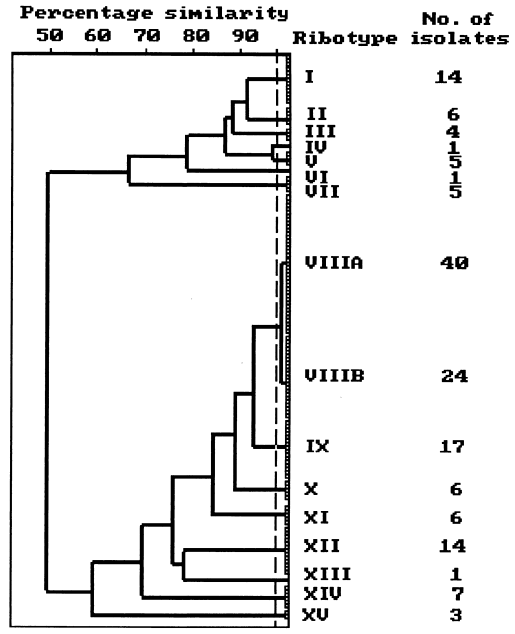

FIG. 3. Dendrogram showing the cluster analysis of rRNA gene restriction fragment profiles of 154 clinical and environmental isolates of *V. cholerae.*

RESULTS

rRNA gene restriction patterns. Analysis of rRNA genes with either *Bgl*I or *Hin*dIII produced reproducible restriction patterns; *Bgl*I produced 16 different patterns and *Hin*dIII produced 4 patterns among the 154 isolates (Fig. 1 and 2). The cleavage patterns consisted of 12 to 16 bands between 27 and 1.5 kb with *Bgl*I and 10 to 12 bands between 10 and 1.2 kb with *Hin*dIII. The *Hin*dIII sites in the rRNA genes are remarkably conserved because bands occurred only at 13 loci, whereas bands occurred at 26 loci in *Bgl*I digests (Fig. 1 and 2).

Cluster analysis and similarity coefficients. A dendrogram representing cluster analysis (Fig. 3) showed that strains belonging to the classical biotype of *V. cholerae* O1 belonged to

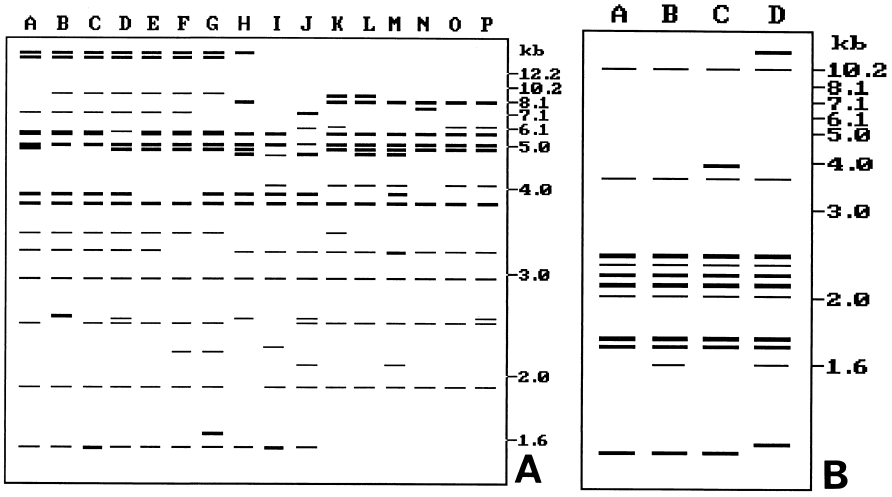

FIG. 2. Schematic diagram of *Bgl*I restriction patterns (A) and *Hin*dIII restriction patterns (B) of the rRNA genes shown in Fig. 1. See text and Fig. 1 legend for details.

TABLE 2. Similarity matrix showing percent similarity[a] among patterns of bands produced by strains belonging to different ribotypes of *V. cholerae*

Ribotype[b]	% Similarity														
	II	III	IV	V	VI	VII	VIIIA	VIIIB	IX	X	XI	XII	XIII	XIV	XV
I[c]	90.6	90.8	92.3	89.2	78.3	76.3	67.9	65.7	62.4	66.0	57.6	58.5	74.2	71.0	51.5
II[c]		84.3	85.6	82.4	80.8	69.7	57.5	58.3	58.1	50.6	53.4	48.9	67.6	60.7	56.7
III[c]			86.1	83.0	84.1	70.4	55.7	53.4	56.3	54.7	51.7	47.4	65.5	77.0	54.9
IV[c]				96.8	73.6	71.9	71.6	69.4	72.3	69.5	67.2	51.0	66.4	62.3	41.4
V[c]					70.7	74.8	68.5	66.2	69.2	66.6	64.2	42.5	63.4	59.2	38.0
VI[c]						58.8	48.1	54.5	48.4	47.4	44.2	40.0	65.7	65.6	53.2
VII[d]							50.8	48.1	50.2	49.6	46.0	34.9	56.1	53.7	27.2
VIIIA[e]								98.5	92.8	90.5	87.1	77.5	74.7	69.6	46.8
VIIIB[f]									91.0	89.0	85.4	75.5	75.7	67.9	48.0
IX[e]										83.6	91.2	69.2	67.0	62.5	38.9
X[e]											81.2	71.9	72.2	65.2	37.8
XI[e]												78.0	76.3	66.5	47.6
XII[e]													76.7	69.1	54.9
XIII[d]														74.7	57.0
XIV[f]															66.9
XV[f]															

[a] Percent similarity on the basis of *Bgl*I and *Hin*dIII restriction patterns of rRNA genes.
[b] Ribotypes XIII to XV were negative for CT and zonula occuludens toxin.
[c] Classical biotype.
[d] *V. cholerae* O139.
[e] El Tor biotype.
[f] *V. cholerae* non-O1 non-O139.

six different ribotypes (ribotypes I to VI), and these were similar at levels between 70.7 and 96.8% (mean similarity coefficient, 0.84). The El Tor vibrios belonged to five different ribotypes (ribotypes VIII to XII) which were similar at a level of between 69.2 and 91.2% (mean similarity coefficient, 0.82). The deepest division revealed by the analysis, at the 47% level of similarity, separated ribotypes I to VII from ribotypes VIII to XV. Table 2 provides a comparison among the different ribotypes in terms of the percent similarity of rRNA gene restriction patterns. The toxigenic O139 vibrios (ribotype VIIIB) were 98.5% similar to a cluster of El Tor vibrios (ribotype VIIIA), but a CT-negative O139 vibrio (ribotype XIII) was similar to the same cluster of El Tor vibrios at a level of only 74.7%. The non-O1 non-O139 strains belonged to three widely different clusters (ribotypes VII, XIV, and XV), with a mean similarity coefficient of 0.49. Strains belonging to ribotype VII were positive for *ctx* and *zot* genes, whereas strains belonging to ribotypes XIV and XV were negative for both toxin genes.

The toxigenic non-O1 non-O139 vibrios belonging to ribotype VII were more similar to ribotypes of classical vibrios (58.8 to 76.3% similarity) than to those of El Tor vibrios (34.9 to 56.1% similarity) and to CT-negative non-O1 non-O139 vibrios (27.2 to 53.7% similarity). The distributions of strains belonging to different ribotypes among the five countries are provided in Table 1. Strains belonging to the same ribotypes were isolated from patients and the environment in the cases of ribotypes VIII through X. All of the CT-positive O139 vibrios isolated from patients in Bangladesh and India and from environmental surface waters also belonged to a single ribotype, designated ribotype VIIIB (Table 1).

DISCUSSION

There have been several studies (5, 6, 21) on the epidemiological application of ribotyping of *V. cholerae* isolates. The present study differs from previous studies in that we have attempted to analyze rRNA gene restriction patterns on a numerical basis and have calculated percent similarities among isolates of different ribotypes. This approach allowed us to calculate a quantity (Table 2) that can be used to express possible clonal relationships between two groups of strains. However, it may be emphasized that the purpose of the present study was not a phylogenetic comparison among strains, because restriction fragment length polymorphism data are not strictly valid for phylogenetic analysis. The similarity values (Table 2) only represent similarities among the different patterns of bands produced by strains belonging to different ribotypes and not overall similarities among the sequences of the rRNA genes carried by the strains.

While the system allows for the use of two or more enzymes to compare rRNA gene patterns for a fine degree of discrimination to enable maximum numbers of strains to be grouped, clusters may be defined with considerable flexibility in terms of percent similarity. The reproducibilities of the *Bgl*I and *Hin*dIII restriction patterns have been tested previously by us and other groups and have been found to be reproducible and stable over time (6, 20). However, we have noticed minor differences among the patterns produced by our assays and those produced by the assay of Popovic et al. (20). These differences could be due to the two different ribotyping probes used in the studies.

Classical *V. cholerae* O1. All classical strains included in the study were isolated in Bangladesh over a 30-year period. These strains consisted of six different ribotypes (Table 1), of which ribotype I strains were most prevalent (14 of 31 strains tested), and were isolated between 1961 and 1968 and between 1982 and 1988. Strains belonging to ribotype I were responsible for a number of epidemics before 1968 and after 1982, and those belonging to ribotype V were responsible for a cholera epidemic in southern Bangladesh in 1989 (5, 26). The association of the strains belonging to the remaining four ribotypes of classical vibrios to epidemics is not clear. The present study confirmed a previous report (5) that strains belonging to one of these ribotypes (ribotype I) associated with epidemics transiently disappeared from Bangladesh after 1968 but that such

strains were again isolated between 1982 and 1992. This, however, is the only example of the reappearance of classical vibrios after apparently being displaced by El Tor vibrios. Nevertheless, this demonstrates the possibility that strains associated with epidemics may survive in ecological shelters and reappear after a long period of time and cause disease.

El Tor *V. cholerae* O1. Of 83 El Tor strains, 38 were from Bangladesh and were isolated between 1969 and 1994, whereas the remaining 45 strains were isolated from four other countries during 1992 and 1993. The Bangladesh isolates belonged to four ribotypes (ribotypes VIIIA, IX, X, and XI), of which isolates of ribotype VIIIA were isolated most frequently and during epidemics (Table 1), and 57.8% of all El Tor strains from Bangladesh tested were ribotype VIIIA. Strains belonging to this ribotype have persisted in Bangladesh for more than 24 years and were isolated from patients or the environment between 1969 and 1993 (Table 1). Ribotype VIIIA strains were also isolated from patients in Nigeria, ribotypes VIIIA and IX were isolated from patients in Syria, and ribotypes VIIIA, IX, and X were isolated from patients in India. All 14 isolates from Tanzania belonged to ribotype XII, and isolates of this ribotype were not isolated from patients in any of the remaining countries. Popovic et al. (20) observed a clone of El Tor vibrios resident only in central African countries, and the reported *Bgl*I restriction patterns of the rRNA genes of these strains appeared to be similar but not identical to those of the Tanzanian strains examined in our study. In the previous study (20) the number of strains from Africa analyzed was very small, and the previous study did not include strains from Tanzania or Nigeria. The present study showed that a unique clone of El Tor vibrios was resident in Tanzania, whereas Nigeria, Syria, and India shared El Tor strains with Bangladesh. However, the number of strains analyzed may not have represented all ribotypes of O1 vibrios circulating in those countries. Nevertheless, the presence of strains in India and Bangladesh belonging to the same ribotypes is consistent with the movement of people across common geographical borders and the sharing of common water sources by the two countries.

***V. cholerae* O139.** The CT-positive O139 vibrios isolated from Bangladesh and India in 1993 belonged to a single ribotype and were 98.5% similar to the most prevalent ribotype (ribotype VIIIA) of El Tor vibrios. In a recent report, Popovic et al. (21) have suggested two closely related ribotypes among the O139 vibrios. This could either be attributed to the difference in the hybridization probe used or to the difference in the sets of O139 isolates tested in the two studies. It should be mentioned that we have previously reported (6) differences in the copy number of *ctx* genes among isolates of O139 vibrios. However, because of the close similarities (>98%) in rRNA gene restriction patterns, we have defined a clone of El Tor vibrios and the O139 vibrios as subribotypes VIIIA and VIIIB, respectively. This is consistent with suggestions that O139 vibrios might have emerged from mutations in serotype-specific genes of an El Tor vibrio (11, 16, 31, 33). However, a heat-stable toxin-positive but CT-negative O139 vibrio isolated from a patient in Argentina (23) (ribotype XIII) was <75% similar to the same cluster of El Tor vibrios (Table 2). This is suggestive of greater heterogeneity among vibrios belonging to the O139 serogroup and more than one possible origin of O139 vibrios.

Comparison among *V. cholerae* O1 and non-O1 isolates. The similarities among the six ribotypes of classical *V. cholerae* O1 isolates in Bangladesh ranged between 70.7 and 96.8%, whereas a toxigenic environmental isolate of non-O1 *V. cholerae* was found to be 76.3 and 74.8% similar to two of the classical ribotype I and IV isolates, respectively (Table 2). The similarity of this clone of non-O1 *V. cholerae* was within the range of divergence of classical ribotypes, and this clone was more closely related to classical vibrios than to El Tor vibrios (34.9 to 56.1% similarity) and other non-O1 nontoxigenic vibrios (27.2 to 53.7% similarity) belonging to ribotypes XIV and XV. The strains of non-O1 *V. cholerae* belonging to ribotype VII were isolated from the environment in 1987 and recently, in 1994 (Table 1). This demonstrated that although classical vibrios belonging to the O1 serogroup are not isolated at present, a toxigenic non-O1 strain closely related to a clone of epidemic classical vibrios (ribotype I) exists in the environment of Bangladesh. Furthermore, this clone of non-O1 vibrios carries both the *ctx* and *zot* genes and presumably the complete *ctx* genetic element (31). Further comparative analysis of the non-O1 strain and classical vibrios is being carried out in our laboratory to characterize other virulence-associated genes and to examine possible evolutionary relationships among the clones.

In view of the clonal variation among both the classical and El Tor vibrios and the emergence of epidemic non-O1 vibrios because of possible mutations in serotype-specific genes in O1 vibrios (11, 33), it is important to characterize toxigenic non-O1 vibrios in the environment.

Comparison of clinical and environmental isolates. Three of the four different ribotypes of El Tor strains found in Bangladesh and the O139 strains were isolated both from patients and from surface water, whereas El Tor strains belonging to the remaining ribotype (ribotype IX) were isolated only from surface water. The isolation of strains belonging to the same ribotypes from the surface water and patients provides further evidence confirming previous indications of the transmission and spread of cholera through surface water in Bangladesh (17).

Our studies so far indicate that strains belonging to ribotype IX have not been isolated from patients with cholera in Bangladesh. However, since strains belonging to ribotype IX were isolated from patients in India in 1992 and from surface water in Bangladesh during December 1994 (Table 1), it is likely that these strains have spread through bodies of water common to the two countries.

We have isolated O139 vibrios from the environment both during the epidemic in 1993 and during a nonepidemic period in 1994, and all of these isolates were found to belong to the same ribotype (Table 2). This demonstrated the persistence of the epidemic clone of O139 vibrios in the environment, and the possibility of further epidemics caused by this clone cannot be ruled out.

Most of the toxigenic *V. cholerae* isolates included in the study were isolated in Dhaka, the capital of Bangladesh. Dhaka is overcrowded, is surrounded by large semirural population centers, and receives an enormous influx of people from rural villages. The growth in the population has far outstripped the capacity of the country to provide adequate housing and sanitation facilities. Although most of the clinical and environmental isolates were from Dhaka, it nevertheless serves as a catchment area for representative strains found throughout the country.

The present study demonstrates that particular ribotypes of toxigenic *V. cholerae* isolates are more often associated with epidemic cholera in Bangladesh. In view of the frequent use of surface waters as potable water supplies in Bangladesh and the evidence indicating transmission of cholera through surface water, it is important to monitor the spread of possible ribotypes responsible for epidemics in environmental surface waters. The study also highlights the importance of comparative analysis of toxigenic non-O1 strains in the environment

with known epidemic strains of O1 or O139 serogroups to monitor the possible emergence of strains with the potential to cause epidemics.

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