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NOTES

Molecular Analysis of rRNA and Cholera Toxin Genes Carried by the New Epidemic Strain of Toxigenic *Vibrio cholerae* O139 synonym Bengal

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***Vibrio cholerae* O139 synonym Bengal recently caused large epidemics of cholera-like disease in Bangladesh and India. We compared the restriction fragment length polymorphisms of *ctxA* and rRNA genes (ribotypes) in 27 isolates of *V. cholerae* O139 from patients in Bangladesh and India with those of 48 isolates of *V. cholerae* O1 from patients and 21 *V. cholerae* isolates from surface waters in Bangladesh, which included 2 O139 and 19 other non-O1 isolates. Ribotyping of the isolates with *BglI* revealed that all 29 isolates of O139 vibrios belonged to a single ribotype, suggesting a clonal nature of the infection. However, the O139 vibrios comprised two *ctxA* genotypes and carried three or more copies of the *ctxA* gene, and the chromosomal locations of these copies were unlike those of the El Tor or classical vibrios. Analysis of the restriction fragment length polymorphisms of the rRNA genes suggested that *V. cholerae* O139 isolates are more closely related to El Tor strains of *V. cholerae* O1 than were 19 other non-O1 vibrios and 33 classical *V. cholerae* O1 isolates that were studied. However, further studies are needed to determine whether *V. cholerae* O139 originated from mutations and genetic changes in a *V. cholerae* O1 strain or was due to the acquisition of virulence genes by a previously unknown *V. cholerae* non-O1 strain.**

In December 1992, an epidemic of severe acute watery diarrhea clinically resembling cholera occurred in southern Bangladesh; it mainly affected adults and later spread to other parts of the country, including the capital city, Dhaka (1, 3). Similar cholera-like outbreaks have also been reported from several places in neighboring India (14). The bacterium responsible for the outbreaks in Bangladesh and India resembled *Vibrio cholerae* O1 in cultural and biochemical characteristics, but did not agglutinate with *V. cholerae* O1 antisera (1, 3, 14). The new epidemic strain of *V. cholerae* was later serogrouped as O139 and was given the suggested name Bengal (3, 17). Primers specific for the cholera toxin (CT) operon from *V. cholerae* O1 amplified sequences corresponding to CT in these strains in PCR (18), and all the strains tested were also positive for CT production by standard bioassays for CT (1, 9, 21).

V. cholerae non-O1 serogroups were not known to be associated with such a large outbreak of diarrhea before the present epidemic. Moreover, they were known to produce CT at a very low frequency (9), unlike the serogroup O139 isolates, for which it was found that all tested isolates produced the toxin. We attempted to characterize the epidemic isolates of *V. cholerae* O139 from the recent outbreaks in Bangladesh and India by analyzing the restriction fragment length polymorphisms (RFLPs) of their rRNA genes and *ctxA* genes to determine the clonal nature of the isolates. The RFLPs of the genes were also compared with those of other *V. cholerae* non-O1 and *V. cholerae* O1 isolates to investigate the relationships among the three groups of isolates and to find out clues about the origin of *V. cholerae* O139.

A total of 29 *V. cholerae* O139 isolates from Bangladesh and India were studied and included 27 clinical and 2 environmental isolates. All non-O1, non-0139 *V. cholerae* isolates and the two environmental isolates of the O139 serogroup were from surface waters in Dhaka, and *V. cholerae* O1 isolates were from patients. The details of the bacteria are given in Table 1. All bacteria were stored in Trypticase soy broth (Difco Laboratories, Detroit, Mich.) with 15% glycerol at −70°C as part of our culture collection.

The identities of all isolates were reconfirmed by standard methods as described previously (3, 23). The RFLPs of the *ctxA* and rRNA genes were studied by hybridizing Southern blots (20) of *BglI*-digested total DNA by using radioactively labeled probes for the *ctxA* and rRNA genes, respectively. The *ctxA* probe was a 550-bp *EcoRI* fragment of pCVD27 (11), and the rRNA gene probe was a 7.5-kb *BamHI* fragment of pKK3535 described previously (2, 5). The recombinant plasmids were prepared and digested with appropriate restriction enzymes (Bethesda Research Laboratories, Gaithersburg, Md.), and the inserts were purified by electroelution from agarose gels (12). The probe DNAs were labeled by random priming (6) by using [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham International plc, Ayelsbury, United Kingdom) and a random primer DNA labeling system (Bethesda Research Laboratories). Southern blots were hybridized, washed under stringent conditions, and autoradiographed as described previously (4,

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TABLE 1. Ribotypes and CT genotypes of *V. cholerae* O1, *V. cholerae* O139, and *V. cholerae* non-O1, non-O139 isolates

Strain	No. of strains	Source	Country	Yr of isolation	Ribotype[a]	*ctxA* genotype[a]
V. cholerae O1						
Classical	4	Patient	Bangladesh	1961–1962	IA	1
Classical	6	Patient	Bangladesh	1963	IB	1
Classical	6	Patient	Bangladesh	1964–1966	IA	1
Classical	1	Patient	Bangladesh	1966	IA	3
Classical	3	Patient	Bangladesh	1967–1968	IA	1
Classical	11	Patient	Bangladesh	1982–1989	IA	1
Classical	2	Patient	Bangladesh	1991	IA	2
El Tor	15	Patient	Bangladesh	1969–1989	IIA	4
V. cholerae non-O1						
O139	18	Patient	Bangladesh	1993	IIB	5
O139	2	Patient	Bangladesh	1993	IIB	6
O139	7	Patient	India	1993	IIB	5
O139	2	S. water[b]	Bangladesh	1993	IIB	5
Non-O139	6	S. water	Bangladesh	1986	III	Negative
Non-O139	1	S. water	Bangladesh	1987	IV	1
Non-O139	1	S. water	Bangladesh	1987	IV	Negative
Non-O139	2	S. water	Bangladesh	1989	V	Negative
Non-O139	9	S. water	Bangladesh	1992–1993	VI	Negative

[a] Ribotypes and *ctxA* genotypes are based on *Bgl*I cleavage patterns.
[b] S. water, surface water.

5). The CT genes in different isolates were further compared by PCR amplification of a 302-bp portion of the CT subunit A gene by using two primers corresponding to nucleotides 712 to 735 and 990 to 1013 of the *ctx* operon of *V. cholerae* O1 (13, 18) and analyzing the PCR products by digestion with the restriction endonucleases *Bam*HI, *Bgl*I, *Eco*RI, *Hin*dIII *Hin*fI, or *Taq*I. The different ribotypes and *ctxA* genotypes were designated on the basis of the differences in the numbers and electrophoretic mobilities of chromosomal bands generated in the Southern hybridization experiments.

All isolates belonging to either serotype O1 or serotype O139 were positive for the CT genes in the PCR assay. Southern blot hybridization of *Bgl*I-digested chromosomal DNAs revealed six different cleavage patterns of the CT genes (CT genotypes 1 through 6; Fig. 1) among the isolates. The cleavage patterns consisted of one to four bands of between 10.5 and 3.2 kb in size. Thirty of the 33 (90.9%) classical *V. cholerae* isolates studied belonged to CT genotype 1, 2 isolates belonged to genotype 2, and 1 isolate belonged to genotype 3. All 15 El Tor isolates belonged to CT genotype 4. Of the 29 isolates of the O139 serotype, 27, including the 2 environmental isolates, belonged to CT genotype 5, and the remaining 2 isolates belonged to genotype 6. Only 1 of the 19 environmental non-O1, non-O139 isolates contained CT genes, and this isolate belonged to genotype 1 (Table 1). The PCR assay produced a 302-bp product for all CT-positive isolates. In agreement with the published sequence of the *ctx* operon (13), the PCR products from all CT-positive strains could be cleaved with *Taq*I and *Hin*fI at the expected positions (see Fig. 3) and did not react with the other four restriction enzymes used.

Ribotyping of the *V. cholerae* isolates produced reproducible restriction patterns, and the strains could be differentiated as belonging to eight different ribotypes on the basis of the *Bgl*I cleavage patterns of their rRNA genes (Fig. 2). The cleavage patterns consisted of 7 to 10 bands between 27 and 1.5 kb for different ribotypes. The classical vibrios comprised two ribotypes, ribotypes IA and IB, whereas all isolates of the El Tor biotype belonged to a single ribotype, designated ribotype IIA. All O139 vibrios isolated from patients in Bangladesh and

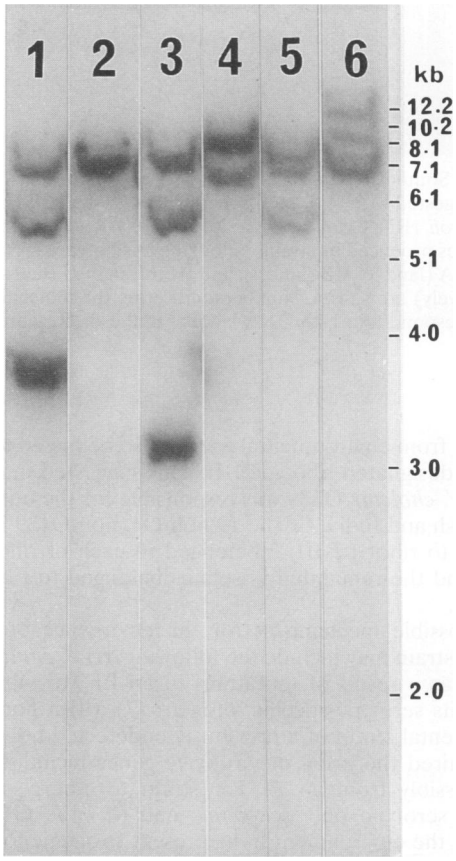

FIG. 1. Southern hybridization analysis of genomic DNA from *V. cholerae* digested with *Bgl*I and probed with a 550-bp fragment of the A subunit of CT derived from pCVD27. *Bgl*I restriction patterns corresponding to CT genotypes 1 through 6 (lanes 1 through 6, respectively) are shown. Numbers indicating the molecular sizes of bands correspond to a 1-kb DNA ladder (Bethesda Research Laboratories).

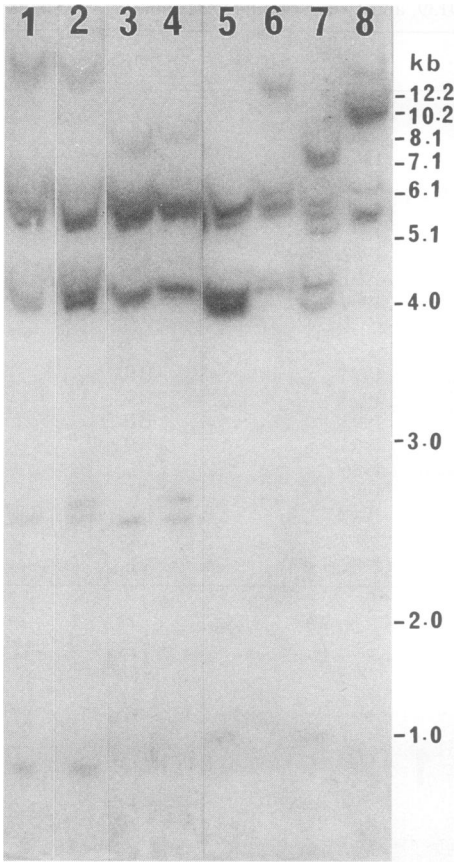

FIG. 2. Southern hybridization analysis of genomic DNA from *V. cholerae* digested with *Bgl*I and probed with a 7.5-kb *Bam*HI fragment of the *E. coli* rRNA clone pKK3535. *Bgl*I restriction patterns of the rRNA genes produced by strains belonging to ribotypes IA (lane 1), IB (lane 2), IIA (lane 3), IIB (lane 4), and III through VI (lanes 5 through 8, respectively) are shown. Numbers indicating the molecular sizes of bands correspond to a 1-kb DNA ladder (Bethesda Research Laboratories).

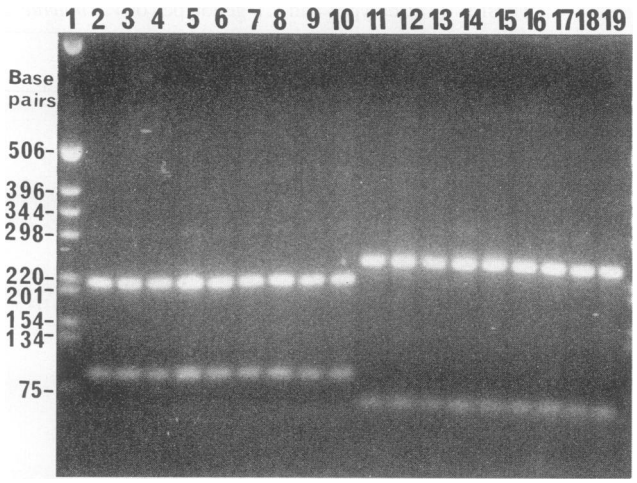

FIG. 3. Restriction analysis of a 302-bp PCR-generated fragment of the *ctxA* gene from *V. cholerae* O1 and O139 strains. The *Hin*fI cleavage patterns of the 302-bp fragment derived from three classical isolates (lanes 2 through 4, respectively), three El Tor isolates (lanes 5 through 7, respectively), and 3 O139 isolates (lanes 8 through 10, respectively) and the *Taq*I cleavage patterns of the 302-bp fragment derived from three classical isolates (lanes 11 through 13, respectively), three El Tor isolates (lanes 14 through 16, respectively), and three O139 isolates (lanes 17 through 19, respectively) are shown. Numbers indicating the molecular sizes of bands correspond to low-molecular-mass fragments of a 1-kb DNA ladder (BRL) are shown in lane 1.

India and from environmental waters also belonged to a single ribotype, designated ribotype IIB. This suggested that a single clone of *V. cholerae* O139 was responsible for the outbreaks in Bangladesh and India. Of the 19 non-O1, non-O139 isolates, 6 belonged to ribotype III, 2 belonged to each of ribotypes IV and V, and the remaining 9 isolates belonged to ribotype VI (Table 1).

The possible mechanisms for the emergence of this new toxigenic strain may include the following: (i) *V. cholerae* O139 emerged as a result of mutations in an El Tor vibrio which changed its serotype-specific antigens (7), (ii) a nontoxigenic environmental strain of a previously undetected non-O1 serotype acquired the array of virulence genes including the CT genes possibly from an El Tor strain to emerge as a new toxigenic serotype of *V. cholerae*, and (iii) the O139 vibrio existed in the environment in its present toxigenic form but in very low numbers and hence was never detected in the past, but some undefined environmental changes have caused the vibrio to multiply rapidly and dominate over existing epidemic strains.

The O139 vibrios belonged to ribotype IIB, which was distinctly different from the ribotypes of the other non-O1 vibrios (ribotypes III through VI) and the classical vibrios (ribotypes IA and IB), but was closely related to that of El Tor vibrios (ribotype IIA). Ribotype IIB differed from ribotype IIA in the *Bgl*I restriction pattern of rRNA genes, which showed an additional band of 2.6 kb in ribotype IIB (Fig. 2). Although the cleavage patterns of the rRNA genes in O139 strains were similar to those of the rRNA genes in El Tor strains, the patterns were not identical. The occurrence of the additional band of 2.6 kb for all O139 strains was very consistent. Moreover, probing of the *Bgl*I restriction fragments of the chromosome for the *ctxA* gene revealed differences among the classical, El Tor, and O139 vibrios. Thus, the differences in the RFLPs of the rRNA and *ctxA* genes between O1 vibrios and O139 vibrios suggest that a mutation(s) in the serotype-specific genes alone of a *V. cholerae* O1 strain cannot account for the emergence of the O139 vibrios, as suggested in a recent report (7). However, the possibility that the O139 vibrios arose as a consequence of a number of mutations and genetic exchanges in an El Tor strain of *V. cholerae* O1 cannot be ruled out.

The 302-bp PCR-generated fragment of the *ctxA* gene from the O1 and O139 isolates showed similar restriction patterns (Fig. 3) and agreed with the published sequence (13) of the *ctxA* gene when tested with a large number of restriction enzymes. This indicates that the 302-bp portion of the *ctxA* gene, if not the entire CT operon, is identical in these strains. The differences in the cleavage patterns of the *ctxA* genes detected by Southern hybridization of total genomic DNAs may have resulted from differences in the copy numbers and chromosomal locations of the genes among the classical, El Tor, and O139 vibrios. This is supported by previous reports indicating that CT genes may be deleted or inserted at different locations within the bacterial genome (22), possibly because of the presence of direct repeat sequences flanking the CT genes (13). Moreover, it has been suggested in a recent report (8) that the *ctx* genes are unusually unstable in the chromosomes of O139 vibrios. This may account for the genetic polymor-

phisms in the *ctxA* genes, and the RFLPs demonstrated may have arisen within the short time span of the epidemic spread of infection. This suggests that the infecting isolates were indeed clonal, as interpreted from the ribotype data and previously reported data on biochemical and cultural characteristics (3), although RFLPs in *ctxA* were observed.

The transformation of a nontoxigenic vibrio to toxigenicity through the acquisition of toxin genes has been suggested previously for the origin of U.S. Gulf Coast El Tor vibrios (22). A similar mechanism may have transformed an ancestral nontoxigenic strain of O139 vibrio which acquired virulence genes from a strain of toxigenic *V. cholerae* O1. However, it is necessary to demonstrate the presence of the ancestral nontoxigenic strain of O139 in the environment to confirm this assumption. In this regard, it is interesting that a CT-negative but heat-stable, toxin-positive *V. cholerae* O139 strain was isolated from a patient with diarrhea in Argentina (15).

Another possibility, that O139 vibrios existed in their present virulent form in very low numbers, and hence were never detected in the past, cannot be ruled out. There are documentations that different clones of toxigenic *V. cholerae* dominated at different times and replaced old epidemic strains (10, 16, 19), and the reasons for the appearance and disappearance of different clones have never been explained. Moreover, the appearance of new clones has sometimes gone undetected because systematic genetic typing methods were not available. We have reported previously (4) the transient appearance of a clone of classical vibrios in 1963 which was not detected either before or after the end of 1963 and suggested that possible undefined environmental changes might influence the emergence and domination of particular clones.

The survival of the new epidemic strain and its domination over O1 strains remain to be monitored in the future. In addition, the possibility remains that more new strains of toxigenic *V. cholerae* with epidemic potential may emerge in the future, and the factors associated with the emergence and domination of epidemic strains need to be identified and monitored.

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REFERENCES

1. **Albert, M. J., A. K. Siddique, M. S. Islam, A. S. G. Faruque, M. Ansaruzzaman, S. M. Faruque, and R. B. Sack.** 1993. A large outbreak of clinical cholera due to *Vibrio cholerae* non-O1 in Bangladesh. Lancet **341**:704.
2. **Brosius, J., A. Ullrich, M. A. Raker, A. Gray, T. J. Dull, R. R. Gutell, and H. F. Noller.** 1981. Construction and fine mapping of recombinant plasmids containing the rrnB ribosomal RNA operon of *E. coli*. Plasmid **6**:112–118.
3. **Cholera Working Group, International Center for Diarrhoeal Disease Research, Bangladesh.** 1993. Large epidemic of cholera like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. Lancet **342**:387–390.
4. **Faruque, S. M., A. R. M. A. Alim, M. M. Rahman, A. K. Siddique, R. B. Sack, and M. J. Albert.** 1993. Clonal relationships among classical *Vibrio cholerae* O1 strains isolated between 1961 and 1992 in Bangladesh. J. Clin. Microbiol. **31**:2513–2516.
5. **Faruque, S. M., K. Haider, M. M. Rahman, A. R. M. A. Alim, Q. S. Ahmad, M. J. Albert, and R. B. Sack.** 1992. Differentiation of *Shigella flexneri* strains by rRNA gene restriction patterns. J. Clin. Microbiol. **30**:2996–2999.
6. **Feinberg, A., and B. Volgelstein.** 1984. A technique for radio labelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **137**:266–267.
7. **Hall, R. H., F. M. Khambaty, M. Kothary, and S. P. Keasler.** 1993. Non-O1 *Vibrio cholerae*. Lancet **342**:430.
8. **Iida, T., J. Shrestha, K. Yamamoto, T. Honda, and M. J. Albert.** 1993. Cholera isolates in relation to the "eighth pandemic." Lancet **342**:925–926.
9. **Janda, J. M., C. Powers, R. B. Bryant, and S. L. Abbott.** 1988. Current perspective on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. Clin. Microbiol. Rev. **1**:245–267.
10. **Kamal, A. M.** 1974. The seventh pandemic of cholera, p. 1–14. *In* D. Barua and W. Burrows (ed.), Cholera. The W. B. Saunders Co., Philadelphia.
11. **Kaper, J. B., J. G. Morris, Jr., and M. Nishibuchi.** 1988. DNA probes for pathogenic *Vibrio* species, p. 65–77. *In* F. C. Tenover (ed.), DNA probes for infectious disease. CRC Press, Inc., Boca, Raton, Fla.
12. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Mekalanos, J. J., D. J. Swartz, G. D. N. Pearson, N. Harford, F. Groyne, and M. Wilde.** 1983. Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. Nature (London) **306**:551–557.
14. **Ramamurthy, T., S. Garg, R. Sharma, S. K. Bhattacharya, G. B. Nair, T. Shimada, T. Takeda, T. Karasawa, H. Kurazano, A. Pal, and Y. Takeda.** 1993. Emergence of a novel strain of *Vibrio cholerae* with epidemic potential in Southern and Eastern India. Lancet **341**:703–704.
15. **Rivas, M., C. Toma, E. Miliwebsky, M. I. Caffer, M. Galas, P. Varela, M. Tous, A. M. Bru, and N. Binsztein.** 1993. Cholera isolates in relation to the "eighth pandemic." Lancet **342**:926–927.
16. **Samadi, A. R., M. I. Huq, N. Shahid, M. U. Khan, A. Eusof, A. S. M. M. Rahman, M. M. Yunus, and A. S. G. Faruque.** 1983. Classical *Vibrio cholerae* biotype displaces El Tor in Bangladesh. Lancet **i**:805–807.
17. **Shimada, T., G. B. Nair, B. C. Deb, M. J. Albert, R. B. Sack, and Y. Takeda.** 1993. Outbreak of *Vibrio cholerae* non-O1 in India and Bangladesh. Lancet **341**:1347.
18. **Shirai, H., M. Nishibuchi, T. Ramamurthy, S. K. Bhattacharya, S. C. Pal, and Y. Takeda.** 1991. Polymerase chain reaction for detection of the cholera enterotoxin operon of *Vibrio cholerae*. J. Clin. Microbiol. **29**:2517–2521.
19. **Siddique, A. K., A. H. Baqui, A. Eusof, K. Haider, M. A. Hossain, I. Bashir, and K. Zaman.** 1991. Survival of classical cholera in Bangladesh. Lancet **337**:1125–1127.
20. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
21. **Spira, W. M., R. B. Sack, and J. L. Froehlich.** 1981. Simple adult rabbit model for *Vibrio cholerae* and enterotoxigenic *Escherichia coli* diarrhea. Infect. Immun. **32**:739–747.
22. **Wachsmuth, I. K., G. M. Evins, P. I. Fields, O. Olsvik, T. Popovic, C. A. Bopp, J. G. Wells, C. Carrilo, and P. A. Blake.** 1993. The molecular epidemiology of cholera in Latin America. J. Infect. Dis. **167**:621–626.
23. **World Health Organization.** 1974. World Health Organization guidelines for the laboratory diagnosis of cholera. Bacterial Disease Unit, World Health Organization, Geneva.