JOURNAL OF CLINICAL MICROBIOLOGY, July 1998, p. 2149–2152 Vol. 36, No. 7
0095-1137/98/$04.00+0

Molecular Epidemiology of Reemergent *Vibrio cholerae* O139 Bengal in India

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Received 10 December 1997/Returned for modification 15 March 1998/Accepted 22 April 1998

We report the prevalence of the O139 serogroup in Calcutta, India, after its reemergence in August 1996 and the spread of the reemerged clone to other parts of the country by using previously established molecular markers. Phenotypically, the reemerged *Vibrio cholerae* O139 displayed a difference compared to those that appeared in late 1992 and 1993 in that the current O139 strains are sensitive to co-trimoxazole. Ribotyping with the enzyme *Bgl*I produced two rRNA restriction patterns in the O139 strains isolated after August 1996, and these patterns were identical to those exhibited by strains of O139 isolated in 1992. Three clones of *V. cholerae* O139 are currently prevailing in the country, with strains exhibiting three bands after *Hin*dIII digestion and hybridization with a *ctxA* probe being dominant. The reemergence of *V. cholerae* O139 in Calcutta after a 32-month quiescent period reestablishes the O139 serogroup as an entity which is likely to play a crucial role in the temporal antigenic variations among the serogroups of *V. cholerae* causing cholera.

Vibrio cholerae is classified into more than 155 serogroups based on the heat-stable somatic O antigen (21, 22). The disease cholera is, however, caused by only two serogroups, namely, O1 and O139. The O139 serogroup is a recent addition which appeared abruptly in September 1992 in southern India (17) and rapidly spread to virtually all areas where cholera is endemic in India (14) and in neighboring countries (15). The extent and rapidity of the spread of the O139 serogroup led us to conclude that this event was the beginning of the eighth pandemic of cholera (14). In February 1994, the O1 serogroup, which had reappeared sporadically in July 1993, again replaced the O139 serogroup and became the dominant serogroup causing cholera in Calcutta, India (11). Subsequent studies showed that the O1 serogroup which replaced the O139 serogroup was a new clone of O1 ElTor biotype (7, 19, 27).

A quiescent period followed in the brief history of *V. cholerae* O139, and it was thought that the appearance of O139 was a one-time event. However, an upsurge of *V. cholerae* O139 was observed in Calcutta in August 1996 (10), and the O139 serogroup again became the dominant serogroup causing cholera by September 1996. Molecular studies showed that the O139 strains which reemerged in August 1996 were indistinguishable from the O139 strains isolated in 1992 and 1993 by ribotyping but showed a unique change in the structure and organization of the CTX genetic element (20). In this study, the prevalence of the O139 serogroup in Calcutta after its reemergence and the spread of the reemerged clone to other parts of the country were investigated by using previously established molecular markers.

The present study is a part of the continuing surveillance program of the National Institute of Cholera and Enteric Diseases, Calcutta, on cholera. Stool specimens were obtained from patients admitted to the Infectious Diseases Hospital, Calcutta, the only hospital which admits cholera patients from the metropolitan city and suburban areas. Methods adopted for collection, transport, bacteriological examination of stool samples, identification, and serotyping of *V. cholerae* have been described in detail previously (11). The National Institute of Cholera and Enteric Diseases, India's national reference laboratory for cholera, and a World Health Organization Reference Centre for Training and Research on Diarrhoeal Diseases, confirms and characterizes *V. cholerae* strains submitted from all over the country and from neighbouring countries. Strains received are characterized by using a panoply of tests as previously described (14). A total of 17 strains of *V. cholerae* O139 isolated between August 1996 and July 1997 from Calcutta and other parts of the country (Table 1) were included in this study for extensive molecular characterization. Another two strains of *V. cholerae* O139, one isolated in 1992 in Calcutta (SG24) and the other isolated in the same year in Madras (MO45 [ATCC 51394]), were included in this study for comparison.

Representative strains of *V. cholerae* O139 isolated from hospitalized patients in Calcutta in 1992 and 1993 (101 strains) and 1996 and 1997 (89 strains) and all 17 representative strains of *V. cholerae* O139 included in the detailed molecular characterization study were examined for resistance to ampicillin (AP) (10 μg), chloramphenicol (30 μg), co-trimoxazole (25 μg), ciprofloxacin (5 μg), furazolidone (FZ) (100 μg), gentamicin (10 μg), neomycin (NM) (30 μg), nalidixic acid (NA) (30 μg), norfloxacin (10 μg), streptomycin (SM) (10 μg), and tetracycline (30 μg) by using commercial discs (Hi Media, Bombay, India) as described previously (11). Characterization of strains as susceptible or resistant was based on size of the inhibition zone around each disc according to the manufacturer's instructions, which matched the interpretive criteria recommended by the World Health Organization (25). Strains showing an intermediate zone of inhibition were interpreted as resistant to that drug on the basis of previous MIC studies conducted with *V. cholerae* (26).

Genomic DNA extraction and Southern hybridization were performed as described earlier (19). Genomic DNA was digested with *Bgl*I for ribotyping and with *Hin*dIII and *Bgl*II for

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TABLE 1. Antibiogram and RFLP analyses of the CTX genetic element of the 17 clinical *V. cholerae* O139 strains isolated from different parts of India after September 1996

Strain no.	Place of isolation	Region	Antibiogram	Restriction pattern[a] of CTX genetic element	Ribotype[b]
AS258	Calcutta	Eastern	AP-FZ-NM-SM	*Hin*dIII: 14.0, 9.0, 7.2; *Bgl*II: 23.0	3a
MD1	Midnapur	Eastern	AP-FZ-NM-SM	*Hin*dIII: 14.0, 9.0, 7.2; *Bgl*II: 23.0	5a
MD3	Midnapur	Eastern	AP-FZ-NM-SM	*Hin*dIII: 14.0, 9.0, 7.2; *Bgl*II: 23.0	3a
NPO549	Nagpur	Deccan	AP-FZ-NM-SM	*Hin*dIII: 14.0, 9.0, 7.2; *Bgl*II: 23.0	3a
YO29	Yavatmal	Deccan	AP-FZ-SM	*Hin*dIII: 14.0, 9.0, 7.2; *Bgl*II: 23.0	3a
MO585	Madras	Southern	AP-FZ-NM-SM	*Hin*dIII: 14.0, 9.0, 7.2	3a
NPO554	Nagpur	Deccan	AP-FZ-SM	*Hin*dIII: 14.0, 9.0, 7.2	3a
NPO564	Nagpur	Deccan	AP-FZ-NM-SM	*Hin*dIII: 14.0, 9.0, 7.2	3a
YO30	Yavatmal	Deccan	AP-FZ-SM	*Hin*dIII: 14.0, 9.0, 7.2	3a
SO58	Sewagram	Deccan	AP-FZ-SM	*Hin*dIII: 14.0, 9.0, 7.2	3a
APO16	Amravati	Deccan	AP-FZ-NM-SM	*Hin*dIII: 14.0, 9.0, 7.2	3a
VO82	Vellore	Southern	AP-FZ-NA-SM	*Hin*dIII: 20.0; *Bgl*II: 8.2, 7.2	3a
VO83	Vellore	Southern	AP-FZ-NA-SM	*Hin*dIII: 20.0; *Bgl*II: 8.2, 7.2	3a
PO42	Pune	Western	AP-FZ-NA-SM	*Hin*dIII: 20.0; *Bgl*II: 8.2, 7.2	5a
WSO10	Solapur	Deccan	AP-FZ-NA-SM	*Hin*dIII: 20.0; *Bgl*II: 8.2, 7.2	5a
BOM6	Bombay	Western	AP-FZ-NM-NA-SM	*Hin*dIII: 15; *Bgl*II: 7.2	5a
MO579	Madras	Southern	AP-FZ-SM	*Hin*dIII: 15	3a

[a] Restriction enzyme and sizes (in kilobases) of the fragments of the CTX genetic element of *V. cholerae* O139 which hybridized with the *ctxA* gene probe.
[b] Ribotype designations are the same as those of Popovic et al. (16).

CTX restriction fragment length polymorphism (RFLP) following the manufacturer's instructions (Boehringer Mannheim GmbH, Mannheim, Germany). Digested fragments were separated by agarose gel electrophoresis (0.8% gel) and Southern hybridized on nylon membrane (Hybond-N^+, Amersham Life Science, Little Chalfont, Buckinghamshire, England). The rRNA gene probe was a 7.5-kb *Bam*HI fragment of pKK3535, which is a pBR322-derived plasmid containing an *Escherichia coli* rRNA operon consisting of one copy each of the genes coding for 5S rRNA, 16S rRNA, 23S rRNA, and $tRNA^{Glu}$ (5). The gene probe for cholera toxin was a 0.5-kb *Eco*RI fragment of pCVD27, which is a pBR325-derived plasmid containing an *Xba*I-*Cla*I fragment representing 94% of the gene encoding the A subunit of cholera toxin (*ctxA*) cloned with *Eco*RI linkers (9). Labelling of the probes, hybridization, and detection of the bands were performed with the ECL detection system (Amersham Life Science) and Kodak Biomax Film (Eastman Kodak Co., Rochester, N.Y.).

The monthly incidence of *V. cholerae* O1 and O139 in patients admitted to the Infectious Diseases Hospital, Calcutta, from January 1996 to December 1997 is shown in Fig. 1. The resurgence of *V. cholerae* O139 in Calcutta began in August 1996, and by September 1996, the O139 serogroup dominated over the O1 serogroup after a lapse of 32 months. After September 1996, O139 strains were also received from different parts of the country. The O139 serogroup dominated until September 1997. From October to December 1997, the O1 serogroup again became the dominant serogroup associated with cholera in Calcutta.

Comparison of the drug resistance patterns between O139 strains isolated in 1992 and 1993 and those isolated in 1996 and 1997 showed that the strains from 1996 and 1997 were suscep-

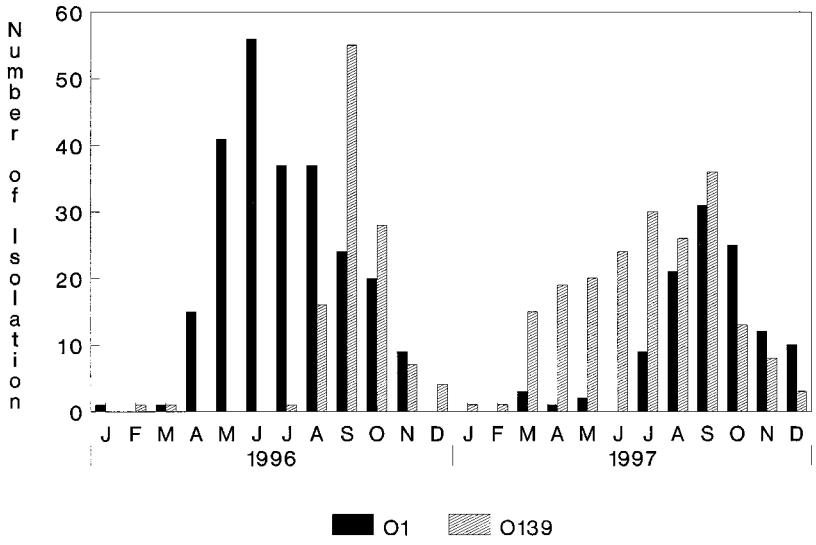

FIG. 1. Monthly isolation of various serogroups of *V. cholerae* from patients hospitalized because of acute secretory diarrhea at the Infectious Diseases Hospital, Calcutta, from January 1996 to December 1997 (J to D, respectively).

tible to co-trimoxazole, unlike the O139 strains from 1992 and 1993, which were resistant to this drug, corroborating our previous observation (10). Interestingly, the O139 strains isolated in 1996 and 1997 are becoming increasingly resistant to ampicillin and neomycin but increasingly susceptible to chloramphenicol and, to a certain extent, streptomycin. The drug resistance pattern of the O139 strains isolated from different parts of the country was similar to the drug resistance pattern of the newly emerged O139 strains in Calcutta with the exception of five strains which were additionally resistant to nalidixic acid (Table 1).

Ribotyping with the enzyme *Bgl*I produced two distinct rRNA restriction patterns in the O139 strains isolated after August 1996 which were similar to the ribotype patterns of the O139 strains isolated in 1992 and 1993 and were designated as 3a and 5a by Popovic et al. (16). The ribotypes 3a and 5a differed from each other by a single 6-kb band. Southern blot analysis of *Hin*dIII-digested genomic DNA of 11 of the 17 O139 strains showed that three bands of 14, 9, and 7.2 kb hybridized with the *ctxA* probe, as reported previously (20). The strains demonstrating this pattern were widely distributed in the country and dominated in the eastern and Deccan regions in India. Two other *Hin*dIII patterns, one in which a 15-kb fragment (shown by two strains) and one in which a 20-kb fragment (shown by four strains) hybridized with the *ctxA* probe, were found sporadically among strains from Southern and Western regions and from a single place in the Deccan region (Table 1). The *Hin*dIII digest showing the 15-kb fragment resembled the pattern shown by SG24 and MO45, the O139 strains which appeared in 1992 (data not shown).

We further analyzed representative strains showing the three different *Hin*dIII patterns with *Bgl*II to understand the structure of the CTX genetic element. Strains which showed three bands after *Hin*dIII digestion showed a single band of about 23 kb when digested with *Bgl*II and probed with *ctxA*, indicating that in these strains the *Bgl*II site resides outside the CTX genetic element, which corroborates our earlier findings (20). Southern blot hybridization of *Bgl*II-digested genomic DNA by using a *ctxA* probe of a representative strain which gave the 15-kb band upon *Hin*dIII digestion showed a single fragment of 7.2 kb. This indicates the presence of a single copy of the CTX genetic element. Southern blot hybridization of *Bgl*II-digested genomic DNA of representative strains which showed a 20-kb *Hin*dIII fragment with a *ctxA* probe demonstrated two fragments of 7.2 and 8.2 kb. This indicates that in these strains there is a tandem duplication of the CTX genetic element.

Until September 1997, the O139 serogroup dominated as the causal serogroup of cholera among hospitalized patients admitted to the Infectious Diseases Hospital, Calcutta. As shown previously (20), the recent O139 strains show substantial reorganization in the structure of the CTX genetic element compared to the 1992 and 1993 O139 strains. In this study, we exploited the unusual *Hin*dIII RFLP of the CTX genetic element of strains of the reemergent O139 serogroup to monitor the spread of this clone to other parts of India. From the results, it is clear that strains of O139 exhibiting three bands after *Hin*dIII digestion of the genomic DNA and subsequently hybridized with *ctxA* probe constitute the dominant O139 clone currently prevailing in the country. This new O139 clone appears to have originated in Calcutta because the representative strain (MO579) of O139 isolated after September 1996 in Madras showed a single 15-kb band after *Hin*dIII digestion, while the representative 1997 Madras strain (MO585) showed three bands like those shown by the Calcutta O139 strains after *Hin*dIII digestion. Therefore, it appears that the new O139 clone may have spread from Calcutta to Madras, which is in contrast to the 1992 situation, when O139 spread from Madras to Calcutta (14, 17).

This study also shows that O139 strains similar to a 1992 clone of O139, which showed a single band of around 15 kb after *Hin*dIII digestion, are prevalent in certain areas, like Bombay. Further restriction analysis with *Bgl*II revealed that this strain had only a single copy of the CTX genetic element. In the 1992 epidemic strains of O139, most strains had two tandemly duplicated copies of the CTX genetic element, while in some a single copy of the element was detected (3, 24). The strains BOM6 and MO579, therefore, appear to be remnants of the O139 clone from 1992 and 1993, which continue to prevail in or represent areas where the new O139 clone has not yet spread. A third *Hin*dIII restriction pattern among O139 strains from Vellore, Pune, and Solapur, India, was observed. Although in these strains there was a duplication of the CTX genetic element, as evident from *Bgl*II digestion, the *Hin*dIII 20-kb band does not resemble the 1992 O139 pattern. Therefore, it is evident that three clones of *V. cholerae* O139 are currently prevailing in the country, with strains exhibiting three bands after *Hin*dIII digestion being dominant.

Phenotypically, the reemerged *V. cholerae* O139 displayed a difference compared to those that appeared in late 1992 and 1993 in that the current O139 strains are sensitive to co-trimoxazole. There was an apparent correlation between antibiogram and *Hin*dIII restriction patterns of the CTX genetic element in the O139 strains examined in this study. The dominant antibiogram of O139 strains displaying three bands after *Hin*dIII digestion and hybridization with a *ctxA* probe was AP-FZ-NM-SM or AP-FZ-SM, while the prominent antibiogram of O139 strains displaying a single 20- or 15-kb *Hin*dIII band was AP-FZ-NA-SM (Table 1). Careful scrutiny of data on multidrug resistance of *V. cholerae* over the past few years indicates that the recent rapid changes being witnessed may be a consequence of the rapid changes in drug resistance of *V. cholerae* (12). In fact, at the time of the genesis of the O139 serogroup, two principal features which distinguished O139 from O1 ElTor strains were the novel O139 serogroup antigen and the distinct pattern of antibiotic resistance (1, 17). While examining the spread of the novel clone of O1 ElTor, we recently observed that the emergence of a new clone of *V. cholerae* O1 was preceded by a change in the antibiogram (2).

Although there were differences in the antibiogram and CTX RFLP between the O139 strains isolated in 1992 and 1993 and in 1996 and 1997, the ribotypes displayed by these temporally spaced-out O139 strains were similar to the ribotypes of the strains isolated in 1992 and 1993. No new ribotype was detected in this study. In contrast, in Bangladesh, differences in ribotypes of O139 strains isolated between 1993 and 1996 have been reported (8, 16). Despite the geographical proximity of and the movement of population between Calcutta and Bangladesh, the epidemiology of cholera can be quite different in the two areas and may be dependent on, among other things, ecological conditions. An outstanding example was the reappearance of the classical biotype of *V. cholerae* in 1983 in Bangladesh (18). The classical biotype of *V. cholerae* O1, however, did not spread to Calcutta or to any other areas where cholera is endemic in India. Within Bangladesh itself, there appears to be a selective distribution of biotypes, with the classical biotype clustering in the southern region while the ElTor biotype prevails in the other regions (23).

The reemergence of *V. cholerae* O139 in Calcutta after a 32-month quiescent period reestablishes the O139 serogroup as an entity which is likely to play a crucial role in the temporal

antigenic variations among the cholera-causing serogroups of *V. cholerae*. The periodic shift between the O1 and O139 serogroups is reminiscent of the shifts from the Ogawa to the Inaba serotypes periodically witnessed among O1 *V. cholerae* in earlier years, possibly mediated by the immune pressure in the population. For some inexplicable reason, the Inaba serotype of the O1 serogroup has disappeared, with the last Inaba dominance being recorded in 1989 in Calcutta (13). The complete disappearance of the Inaba serotype from Calcutta strangely coincides with the genesis of the O139 serogroup. Molecular studies have now shown that the O139 serogroup originated from an O1 biotype ElTor strain by acquisition of novel DNA which replaced the *rfb* genes encoding the O1 antigen (4, 6). Epidemiologically, it appears that the O139 serogroup has appeared as an alternate to the Inaba serotype of the O1 serogroup to aid the persistence and perpetuate the spread of cholera.

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