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

ASISH KUMAR MUKHOPADHYAY,1 ARNAB BASU,1 PALLAVI GARG,1 PRASANTA K. BAG,1 AMIT GHOSH,2 S. K. BHATTACHARYA,1 YOSHIFUMI TAKEDA,3 AND G. BALAKRISH NAIR1*

National Institute of Cholera and Enteric Diseases, Beliaghata, Calcutta-700010, and Institute of Microbial Technology, Chandigarh,2 India, and Research Institute, International Medical Center of Japan, Shinjuku-ku, Tokyo 162, Japan3

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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* II 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 *Hind* III 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* II for ribotyping and with *Hind* III and *Bgl* II for...
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\textsuperscript{1}, Amersham Life Science, Little Chalfont, Buckinghamshire, England). The rRNA gene probe was a 7.5-kb \textit{Bam}HI fragment of pKK3535, which is a pBR322-derived plasmid containing an \textit{Escherichia coli} rRNA operon consisting of one copy each of the genes coding for 5S rRNA, 16S rRNA, 23S rRNA, and tRNAGlu (5). The gene probe for cholera toxin was a 0.5-kb \textit{Eco}RI fragment of pCVD27, which is a pBR325-derived plasmid containing an \textit{Xba}\textit{I}-\textit{Cla}\textit{I} fragment representing 94% of the gene encoding the A subunit of cholera toxin (\textit{ctxA}) cloned with \textit{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 \textit{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 \textit{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-

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

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Place of isolation</th>
<th>Region</th>
<th>Antibiogram</th>
<th>Restriction pattern\textsuperscript{a} of CTX genetic element</th>
<th>Ribotype\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS258</td>
<td>Calcutta</td>
<td>Eastern</td>
<td>AP-FZ-NM-SM</td>
<td>\textit{HindIII}: 14.0, 9.0, 7.2; \textit{BglII}: 23.0</td>
<td>3a</td>
</tr>
<tr>
<td>MD1</td>
<td>Midnapur</td>
<td>Eastern</td>
<td>AP-FZ-NM-SM</td>
<td>\textit{HindIII}: 14.0, 9.0, 7.2; \textit{BglII}: 23.0</td>
<td>5a</td>
</tr>
<tr>
<td>MD3</td>
<td>Midnapur</td>
<td>Eastern</td>
<td>AP-FZ-NM-SM</td>
<td>\textit{HindIII}: 14.0, 9.0, 7.2; \textit{BglII}: 23.0</td>
<td>3a</td>
</tr>
<tr>
<td>NPO549</td>
<td>Nagpur</td>
<td>Deccan</td>
<td>AP-FZ-NM-SM</td>
<td>\textit{HindIII}: 14.0, 9.0, 7.2; \textit{BglII}: 23.0</td>
<td>3a</td>
</tr>
<tr>
<td>YO29</td>
<td>Yavatmal</td>
<td>Deccan</td>
<td>AP-FZ-SM</td>
<td>\textit{HindIII}: 14.0, 9.0, 7.2</td>
<td>3a</td>
</tr>
<tr>
<td>MO585</td>
<td>Madras</td>
<td>Southern</td>
<td>AP-FZ-NM-SM</td>
<td>\textit{HindIII}: 14.0, 9.0, 7.2</td>
<td>3a</td>
</tr>
<tr>
<td>NPO554</td>
<td>Nagpur</td>
<td>Deccan</td>
<td>AP-FZ-SM</td>
<td>\textit{HindIII}: 14.0, 9.0, 7.2</td>
<td>3a</td>
</tr>
<tr>
<td>NPO564</td>
<td>Nagpur</td>
<td>Deccan</td>
<td>AP-FZ-NM-SM</td>
<td>\textit{HindIII}: 14.0, 9.0, 7.2</td>
<td>3a</td>
</tr>
<tr>
<td>YO30</td>
<td>Yavatmal</td>
<td>Deccan</td>
<td>AP-FZ-SM</td>
<td>\textit{HindIII}: 14.0, 9.0, 7.2</td>
<td>3a</td>
</tr>
<tr>
<td>SO58</td>
<td>Sewagram</td>
<td>Deccan</td>
<td>AP-FZ-SM</td>
<td>\textit{HindIII}: 14.0, 9.0, 7.2</td>
<td>3a</td>
</tr>
<tr>
<td>APO16</td>
<td>Amravati</td>
<td>Deccan</td>
<td>AP-FZ-NM-SM</td>
<td>\textit{HindIII}: 14.0, 9.0, 7.2</td>
<td>3a</td>
</tr>
<tr>
<td>V082</td>
<td>Vellore</td>
<td>Southern</td>
<td>AP-FZ-NA-SM</td>
<td>\textit{HindIII}: 20.0; \textit{BglII}: 8.2, 7.2</td>
<td>3a</td>
</tr>
<tr>
<td>V083</td>
<td>Vellore</td>
<td>Southern</td>
<td>AP-FZ-NA-SM</td>
<td>\textit{HindIII}: 20.0; \textit{BglII}: 8.2, 7.2</td>
<td>3a</td>
</tr>
<tr>
<td>PO42</td>
<td>Pune</td>
<td>Western</td>
<td>AP-FZ-SM</td>
<td>\textit{HindIII}: 20.0; \textit{BglII}: 8.2, 7.2</td>
<td>5a</td>
</tr>
<tr>
<td>WSO10</td>
<td>Solapur</td>
<td>Deccan</td>
<td>AP-FZ-NM-SM</td>
<td>\textit{HindIII}: 20.0; \textit{BglII}: 8.2, 7.2</td>
<td>5a</td>
</tr>
<tr>
<td>BOM6</td>
<td>Bombay</td>
<td>Western</td>
<td>AP-FZ-NM-NA-SM</td>
<td>\textit{HindIII}: 15; \textit{BglII}: 7.2</td>
<td>5a</td>
</tr>
<tr>
<td>MO579</td>
<td>Madras</td>
<td>Southern</td>
<td>AP-FZ-SM</td>
<td>\textit{HindIII}: 15</td>
<td>3a</td>
</tr>
</tbody>
</table>

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

FIG. 1. Monthly isolation of various serogroups of \textit{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).
HindIII 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 HindIII digestion, are prevalent in certain areas, like Bombay. Further restriction analysis with BglII revealed that this strain had only a single copy of the CTX genetic element. In 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 HindIII 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 BglII digestion, the HindIII 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 HindIII 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 HindIII 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 HindIII 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 HindIII 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 ribotypes, with the classical biotype clustering in the southern region, while the ElTor biotype prevails in the other regions (25).

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 El Tor 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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