Molecular Analysis of Non-O1, Non-O139 Vibrio cholerae Associated with an Unusual Upsurge in the Incidence of Cholera-Like Disease in Calcutta, India

CHARU SHARMA,1 M. THUNGAPATHRA,1 A. GHOSH,1 ASISH K. MUKHOPADHYAY,2 ARNAB BASU,2 RUPAK MITRA,1 INDIRA BASU,2 S. K. BHATTACHARYA,2 T. SHIMADA,3 T. RAMAMURTHY,4 T. TAKEDA,5 S. YAMASAKI,5 Y. TAKEDA,5 AND G. BALAKRISH NAIR2a

Institute of Microbial Technology, Chandigarh,1 and National Institute of Cholera and Enteric Diseases, Calcutta,2 India, and Laboratory of Enteric Infection 1, National Institute of Infectious Diseases,3 and Research Institute, International Medical Center of Japan,4 Tokyo 162, and Department of Infectious Diseases Research, National Children’s Medical Research Center, Tokyo 154,4 Japan

Received 17 June 1997/Returned for modification 22 October 1997/Accepted 15 December 1997

There was an inexplicable upsurge in the incidence of non-O1, non-O139 Vibrio cholerae among hospitalized patients admitted to the Infectious Diseases Hospital, Calcutta, India, between February and March 1996. Of the 18 strains of V. cholerae isolated during this period, 15 belonged to the non-O1, non-O139 serogroups (4 belonged to O144, 3 belonged to O11, 1 each belonged to O6, O8, O12, O19, O39, and O38, and 2 strains could not be typed), 2 belonged to the O139 serogroup, and 1 belonged to the O1 serogroup. Cell-free culture supernatants of 13 representative non-O1, non-O139 V. cholerae strains evoked a distinct cytotoxic effect on CHO and HeLa cells, and the strains examined produced the nonmembrane-damaging cytotoxin. By several PCR assays, it was determined that none of the non-O1, non-O139 strains were positive for the ctxA, zot, ace, and tcpA genes and for the genes representing the heat-labile toxin, heat-stable toxin, and verotoxin of Escherichia coli and the various variants of these genes. Studies on the clonality of non-O1, non-O139 V. cholerae strains by restriction fragment length polymorphism (RFLP) analysis of rRNA genes and of other genes (hlyA, hlyC, hlyU, hls, toxR, and attR51) and by pulsed-field gel electrophoresis (PFGE) collectively indicate that the upsurge which occurred in February and March 1996 was caused by strains belonging to different clones. Overall, there was an excellent correlation between the results of ribotyping, RFLP analysis of various genes, and PFGE, with strains belonging to a particular serogroup showing nearly identical restriction patterns and PFGE profiles. It is clear from this study that some serogroups of V. cholerae can cause diarrhea by a mechanism quite different from that of toxigenic V. cholerae O1 and O139, and we have proposed the nomenclature of enteropathogenic V. cholerae to include these serogroups.

Vibrio cholerae strains belonging to serogroups O1 and O139 are the causative agents of cholera, while the non-O1, non-O139 serogroups of V. cholerae comprise a heterogeneous group of organisms whose clinical association with humans is inadequately understood. Clinically, apart from the O1 and O139 serogroups, the non-O1, non-O139 serogroups continue to be of negligible significance since these strains are associated with illness in only a low percentage of patients hospitalized due to acute secretory diarrhea (18). Nucleotide analysis of the asd genes of 45 strains of V. cholerae has yielded provocative evidence which indicates that the classical and El Tor biotypes and U.S. Gulf Coast strains of V. cholerae O1 evolved independently from environmental nonenteric, non-O1, non-O139 strains (15). Therefore, it has become increasingly clear that the non-O1, non-O139 serogroups are involved in the emergence of newer variants of V. cholerae, a fact supported by the genetics of V. cholerae O139, which is believed to have evolved as a result of horizontal gene transfer between the O1 and the non-O1 serogroups (4).

These recent events have led us to fortify our cholera surveillance program in Calcutta, India, and to extend our monitoring to the non-O1, non-O139 serogroups as well. While conducting this survey, we observed an inexplicable rise in the incidence of non-O1, non-O139 V. cholerae in February and March 1996 among hospitalized patients admitted to the Infectious Diseases (ID) Hospital in Calcutta. In fact, the rate of isolation of non-O1, non-O139 strains of V. cholerae exceeded that of O1 and O139 serogroups during the period mentioned above. In this study, we report the extensive molecular characterization of the non-O1, non-O139 strains isolated between February and March 1996 from hospitalized patients admitted to the ID Hospital, Calcutta.

MATERIALS AND METHODS

Bacteriology and serogrouping. This study was conducted among hospitalized patients admitted to the ID Hospital, Calcutta, the only hospital which admits cholera patients from metropolitan Calcutta and surrounding areas. Upon admission, a thorough clinical evaluation with particular attention to the degree of dehydration was conducted and a retrospective history was recorded in a standard proforma manner. Stool samples or rectal swabs collected in Cary Blair medium were processed in the laboratory within 2 h of collection for the isolation of V. cholerae and other enteropathogens by previously published techniques (18, 36). A multitest medium was used for the presumptive identification of V. cholerae (14, 20). All strains were subsequently examined for the oxidase reaction, and the identity of V. cholerae O1 was confirmed by serogrouping, using growth from the multitest medium, with polyvalent O1 and monospecific Inaba and Ogawa antisera raised at the National Institute of Cholera and Enteric Diseases, Calcutta. V. cholerae strains which did not agglutinate with the O1 antisem were checked with monoclonal O139 antiserum developed at the National Institute of Cholera and Enteric Diseases (9). V. cholerae strains which did not agglutinate with either O1 or O139 antisem were assumed to belong to the non-O1 and non-O139 serogroups, and these strains were further serogrouped.

* Corresponding author. Mailing address: National Institute of Cholera and Enteric Diseases, P-33, CIT Road, Scheme XM, Beliaghata, Calcutta 700 010, India. Phone: 91-33-3504598. Fax: 91-33-3505066. E-mail: krishgb@giacs01.vsnl.net.in.
TABLE 1. Sequences of primers used for detection of different genes by the PCR assay

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Amplification (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ctcA</td>
<td>TCT AGA CGG ATT TTA TAG ACG GCC ACC</td>
<td>301</td>
<td>16</td>
</tr>
<tr>
<td>TCT ACT TTC GTA GCC CCT ATT ACG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>zot</td>
<td>CAC TGT TGA GCG TTA TCG</td>
<td>243</td>
<td>6</td>
</tr>
<tr>
<td>CAA GGG CTT TGG GTA GAA GGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ace</td>
<td>GCT TAG GAT GCA CCC TTA</td>
<td>284</td>
<td>6</td>
</tr>
<tr>
<td>TTT GGC CTT CGA GGA TAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tcpA (El)</td>
<td>GAA GAA GGT TAA AAG AGA ACA A</td>
<td>471</td>
<td>16</td>
</tr>
<tr>
<td>Tor</td>
<td>GAA AGG ATC TTT CAT CTT G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tcpA (classical)</td>
<td>CAC GAT AAG AAA ACC GGT CAT CAG GAA G</td>
<td>617</td>
<td>16</td>
</tr>
<tr>
<td>ACC AAA TGC ACC GGA TGG AG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTe</td>
<td>GCG ACA AAT TAG ACC GTT CG</td>
<td>708</td>
<td>33</td>
</tr>
<tr>
<td>CAG AAT TCT GTT ATA TAG GT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stc</td>
<td>ATT TTT C/ATT TCT GTA TAG TGC</td>
<td>105</td>
<td>30</td>
</tr>
<tr>
<td>GC/TA CAG/A GCA GTA TAA CAA AA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vtc</td>
<td>GAA CGA AAT AAT TAT TAT GTG</td>
<td>521</td>
<td>32</td>
</tr>
<tr>
<td>TGA TAG TGA CAA TAG AGT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

by the somatic O-antigen serogrouping scheme for V. cholerae developed at the National Institute of Infectious Diseases, Tokyo, Japan (29).

The tissue culture assay. The non-O1, non-O139 V. cholerae strains isolated during the study period were examined by tissue culture assay with CHO and HeLa cells. The strains were grown in Trypticase soy broth (TSB; Difco, Detroit, Mich.) supplemented with 0.6% yeast extract (TSB-YE) and in AKI medium containing 10% (vol/vol) horse serum (Gibco Laboratories, Grand Island, N.Y.). Cell lines were maintained in 25-cm² tissue culture flasks (NUNC, Roskilde, Denmark) with 10% (vol/vol) horse serum (Gibco Laboratories, Grand Island, N.Y.). Cells were incubated as described above. Morphological changes in CHO and HeLa cells were examined for the presence of heat-labile toxin (LT), heat-stable toxin (ST), and verotoxin (VT) of Escherichia coli and its variants with primers common to the toxin and the variants. The primers used for this assay and the expected amplicon sizes are listed in Table 1. The following were added to each 100 µl of PCR mixture: 10 µl of Mg-free PCR mixture (50 mM KCl, 100 mM Tris HCl [pH 9.0], 0.1% Triton X-100); 8 µl of 25 mM MgCl₂; 2 µl each of 2 mM dATP, dTTTP, dGTP, and dCTP; 50 pmol each of the primers; and 2.5 µl of Taq DNA polymerase (Takara Shuzo, Otsu, Japan). PCR was carried out in 0.5-ml microcentrifuge tubes with 43.5 µl of the PCR mixture described above and 6.5 µl of a Luria broth (Difco) culture of bacteria heated at 94°C for 5 min. The solution was overlaid with a drop of sterile mineral oil (Sigma), and PCR was performed in an automated thermocycler (UNO-Thermoblock; Biometra, Göttingen, Germany) for 30 cycles, and the cycling conditions were as follows: denaturation at 94°C for 1.5 min, annealing at 60°C (but in the case of zot and ace the annealing temperature was 55°C and for LtC, VTc, and Stc the annealing temperature was 50°C), and extension at 72°C for 1.5 min. A reagent blank (containing all the components of the reaction mixture and water instead of broth containing template DNA) and strains VC20 (V. cholerae O1 El Tor Ogawa), S69B (V. cholerae O1 classic Inaba), B2C (LT- and ST-positive strain of E. coli), and T17 (VT-positive strain of E. coli) were run as controls. Amplified products from the PCR were electrophoresed on 2.5% agarose gels and were stained with ethidium bromide. A 1-kb molecular size ladder (Gibco BRL, Gaithersburg, Md.) was run with each gel.

Preparation of DNA probes, Southern blotting, and DNA hybridization. The DNA probes for hlyC, hlyA, and toxR were a 0.769-kb EcoRI fragment, a 1.7-kb EcoRI fragment, and a 0.9-kb XbaI-SalI fragment, respectively, from plasmids pHU2, pGtT9, and pToxR II, respectively. These probes were amplified by PCR for V. cholerae 5989B O1 chromosomal DNA as the template and with various oligonucleotide primers, which are listed in Table 1. The probes used for this work were specific for toxA in all the strains tested. The probes for the tcpA gene were a 29-base oligonucleotide (5'-GGT CTG CTC ACT GCT TGA GCC CAA GA-3') located in the middle of the 17-bp coding region (19). The attR51 probe was an 18-base oligonucleotide (5'-CCT TAG TGC GTA TTA GTG-3') corresponding to the 17-bp target sequence termed attRS1, in which the RS1 of the CTX genetic element integrates into the chromosome of V. cholerae strains (22). The 13-O1, non-O139 V. cholerae strains listed in Table 3 were analyzed for restriction fragment length polymorphisms (RFLPs) of hlyC, hlyA, hly, attR51, and toxR, respectively. Chromosomal DNA was prepared as described earlier (21). Samples of 2 µg of the DNA preparations were digested with a variety of restriction endonucleases (Promega, Madison, Wis.) according to the manufacturer’s instruction. The restricted fragments were separated by electrophoresis through 0.7% (wt/vol) gels, and Southern hybridization was performed as described before (21). Probes were labeled with [32P]dATP either by nick translation by or 5'-end linking with polynucleotide kinase (25).

PFGE. For the pulsed-field gel electrophoresis (PFGE) study, 16 strains of V. cholerae were analyzed. These included nine of the non-O1, non-O139 V. cholerae strains associated with the upsurge in Calcutta (the remaining six strains could not be analyzed by PFGE due to endonuclease activity), 3 strains of non-O1, non-O139 V. cholerae belonging to serogroups O60 (strain S2G), O2 (strain S2G), and O37 (strain SG69) associated with sporadic infections and isolated in 1992 from hospitalized patients, one strain of Vibrio fluvialis (strain AS63), representing a species other than V. cholerae isolated from a patient with diarrhea in the same time frame as the non-O1, non-O139 upsurge, and three reference strains of toxigenic V. cholerae (5). The hemolysis in V. cholerae enterotoxin gene probe and was used to develop a method for the preparation of DNA preparations of the toxigenic strains used for the PFGE studies (strain S69B). One O1 El Tor biotype (strain V5), and the O139 serogroup (strain SG26), respectively.

To perform PFGE, the genomic DNA of the various strains was prepared by agarose plugs as described previously (17). Agarose blocks containing genomic DNA were equilibrated in restriction enzyme buffer for 1 h at room temperature and were cleaved in fresh buffer at the appropriate incubation temperature. For complete digestion of the DNAs, 50 U of NorI was used. PFGE of the inserts was performed by the contour-clamped homogeneous electric field method on a CHEF DR-II apparatus (Bio-Rad, Richmond, Calif.) in 0.4× TBE buffer (44.5 µl of 2× TBE buffer, 100 µl of 10× TBE buffer, and 100 µl of water). Digestions were electrophoresed at a field strength of 60 V/cm. Pulse times were increased from 0.5 min to 30 min in 10 min increments. Data were recorded on a video cassette recorder. The PFGE gel images were analyzed using Quantity One software (Bio-Rad Laboratories, Hercules, Calif.). The size of the DNA fragments was estimated by the position of the 1-kb molecular size marker (Invitrogen, San Diego, Calif.). The results of the PFGE analysis were compared with the PFGE patterns of the non-O1, non-O139 V. cholerae strains listed in Table 3.
mM Tris-HCl, 44.5 mM boric acid, 1.0 mM EDTA [pH 8.0]). The gels were electrophoresed for 19.5 h with pulse times of 5 to 35 s, and electrophoresis was continued for 23.5 h with pulse times of 6.72 to 17.3 s. A DNA size standard (bacteriophage λ ladder; Bio-Rad) was used as the molecular mass standard, and a model 1000 mini-chiller (Bio-Rad) was used to maintain the temperature of the buffer at 14°C. The gels were stained in distilled water containing 1.0 μg of ethidium bromide per ml for 30 min, rinsed several times in tap water, and photographed under UV light.

**Interpretation of PFGE patterns.** The picture obtained from PFGE was directly scanned on a model 420 optically enhanced densitometer-scanner interfaced with a computer in which the program Diversity One (version 1.6) was installed. Comparisons of differences in the patterns of NotI-digested DNA were made to ascertain the phylogenetic relationship between strains by using the software that runs on a Sun workstation (pdi Inc., Huntington Station, N.Y.). A phylogenetic tree was made with the average percentage of matched bands representing LT, ST, and VT of *V. cholerae* non-O1, non-O139.

### RESULTS

In 1996, an unusual event occurred in the beginning of the cholera season in Calcutta. From the end of February 1996, there was an inexplicable upsurge in the incidence of non-O1, non-O139 *V. cholerae* infections among hospitalized patients admitted to the ID Hospital (Fig. 1). The incidence of the non-O1, non-O139 serogroup exceeded the incidence of the O1 or the O139 serogroup of *V. cholerae* during this period for the first time since we initiated the focused cholera surveillance program in Calcutta 141 months earlier. A total of 18 strains of *V. cholerae* were isolated from 18 of the 232 patients examined during February and March 1996. For all 18 patients, *V. cholerae* was the sole pathogen isolated. Of these 18 isolates, 15 belonged to non-O1, non-O139 serogroups, 2 belonged to the O139 serogroup, and 1 belonged to the O1 serogroup. Serotyping of the 15 non-O1, non-O139 strains revealed that 4 belonged to O144, 3 belonged to O11, 1 each belonged to O6, O8, O12, O19, O39, and O58, and 2 strains could not be typed. The antibiotic susceptibilities of the 13 non-O1, non-O139 *V. cholerae* strains isolated in this study are presented in Table 3. All strains were resistant to multiple antibiotics with the *V. cholerae* strains belonging to serogroup O144 being resistant to more than seven of the antibiotics used in this study.

All the patients excreting the non-O1, non-O139 *V. cholerae* strains developed severe dehydration with hypovolemic shock after a short period of diarrhea (mean preadmission duration of less than 12 h) and vomiting. The patients required large amounts of intravenous fluids for correction of the initial dehydration and hypovolemic shock. The postadmission duration of purging was about 35 h. All the patients were successfully rehydrated with intravenous and oral fluids, and there was no mortality in this series.

The 13 non-O1, non-O139 *V. cholerae* strains associated with cholera-like disease were further characterized in extensive detail to obtain an understanding of the virulence traits which might have contributed to the disease. As indicated in Table 3, cell-free culture supernatants of all of the strains grown in TSB-YE and AKI medium evoked a distinct cytotoxic effect on CHO and HeLa cells, while culture supernatants of the strains examined also produced NMDCY when they were cultured in AKI medium. In the PCR assay, all the strains yielded negative results for the *ctxA*, *zot*, *ace*, and *tcpA* genes. Furthermore, all non-O1, non-O139 strains were negative by PCR for genes representing LT, ST, and VT of *E. coli* and its various variants and also did not hybridize with a DNA probe specific for NAG-ST. Further analysis of 13 of the non-O1, non-O139 *V. cholerae* strains showed that all strains hybridized with DNA probes specific for the El Tor hemolysin (*hlyA*), for the regulatory gene *hlyU* which upregulates expression of the El Tor hemolysin (34), and with the oligonucleotide probe specific for the recently described *hlt* gene. In addition, all the strains hybridized with the DNA probe specific for *toxA* and with an oligonucleotide probe for the specific 17-bp target sequence termed *attRS1*.

We further examined the genetic relatedness between the various strains of non-O1, non-O139 *V. cholerae* isolated between February and March 1996 by investigating the RFLPs of the *hlyA*, *hlyU*, *hlt*, *toxA*, *attRS1*, and tRNA genes. A single *PstI* fragment of 6 kb hybridized with the *hlyA* gene probe for all strains examined, as indicated in Fig. 2A. RFLP analysis of the *hlyU* gene with XbaI-*BglII* showed two patterns designated HUI and HUII (Fig. 2B; Table 3) among the strains examined. Pattern HUI consisted of only one band of 3.3 kb, while pattern HUII consisted of four bands of 6.3, 5.0, 4.3, and 3.3 kb. RFLP analysis of the *hlt* gene with *Sau3A1* displayed two profiles designated HLI and HLII (Fig. 2C; Table 3), with profile HLI consisting of a band of 0.568 kb and HLII consist-
ing of a band of 0.7 kb. RFLP analysis of the attRS1 target sequence with HindIII displayed two main profiles designated A1 and A2. Strains AM109, AM111, AM112, and AM113 displayed unique RFLP patterns, while strain AS66 showed partial digestion, and therefore, multiple bands were seen (Fig. 2D; Table 3). Finally, RFLP analysis of the toxR gene with PstI showed three patterns (TR1, TR2, and TR3) among the strains examined (Fig. 2E). Ribotyping of the 13 non-O1, non-O139 V. cholerae strains examined with BglI produced six patterns which were designated R1 to R6 (Fig. 3). All strains belonging to serogroup O144 were found to have pattern R1, while the strains belonging to O11 and OUT (O untypeable) had pattern R2. The remaining four strains showed polymorphisms in their ribotype patterns. None of the six ribotypes matched any of the patterns documented for the standardized ribotypes documented for V. cholerae by Popovic et al. (23). The PFGE profiles of the V. cholerae strains obtained with NorI showed a variety of patterns which correlated well with

![Table 3. Characteristics of non-O1, non-O139 V. cholerae strains isolated during February and March 1996 in Calcutta](image)

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Date of isolation (day.mo.yr)</th>
<th>Sero-group</th>
<th>Cleavage pattern of the following genes:</th>
<th>Ribotype</th>
<th>NMDCY</th>
<th>Tissue culture activity</th>
<th>Antibiogram</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM107</td>
<td>29.2.1996</td>
<td>O144</td>
<td>HUII HLIi A1 TR1 R1</td>
<td>1.43</td>
<td>Ct</td>
<td>Ct</td>
<td>A Ct Co Fz N Na Ns S</td>
</tr>
<tr>
<td>AM108</td>
<td>29.2.1996</td>
<td>O144</td>
<td>HUII HLIi A1 TR1 R1</td>
<td>2.15</td>
<td>Ct</td>
<td>Ct</td>
<td>A Ct Co Fz N Na Ns S</td>
</tr>
<tr>
<td>AS60</td>
<td>29.2.1996</td>
<td>O144</td>
<td>HUII HLIi A1 TR1 R1</td>
<td>1.59</td>
<td>Ct</td>
<td>Ct</td>
<td>A Ct Co Fz N Na Ns S</td>
</tr>
<tr>
<td>AS61</td>
<td>29.2.1996</td>
<td>O144</td>
<td>HUII HLIi A1 TR1 R1</td>
<td>1.51</td>
<td>Ct</td>
<td>Ct</td>
<td>A Ct Co Fz N Na Ns S</td>
</tr>
<tr>
<td>AM109</td>
<td>14.3.1996</td>
<td>O19</td>
<td>HUII HLIi UP TR3 R3</td>
<td>1.82</td>
<td>Ct</td>
<td>Ct</td>
<td>A Fz N S</td>
</tr>
<tr>
<td>AM111</td>
<td>18.3.1996</td>
<td>O12</td>
<td>HUII HLIi UP TR3 R3</td>
<td>ND</td>
<td>Ct</td>
<td>Ct</td>
<td>A Fz N Na</td>
</tr>
<tr>
<td>AM112</td>
<td>19.3.1996</td>
<td>O39</td>
<td>HUII HLIi UP TR2 R5</td>
<td>2.13</td>
<td>Ct</td>
<td>Ct</td>
<td>A Fz N S</td>
</tr>
<tr>
<td>AM113</td>
<td>20.3.1996</td>
<td>O6</td>
<td>HUII HLIi UP TR2 R6</td>
<td>2.46</td>
<td>Ct</td>
<td>Ct</td>
<td>A Fz N S</td>
</tr>
<tr>
<td>AS64</td>
<td>21.3.1996</td>
<td>O11</td>
<td>HUII HLIi A2 TR3 R2</td>
<td>1.21</td>
<td>Ct</td>
<td>Ct</td>
<td>A Fz N S</td>
</tr>
<tr>
<td>AS65</td>
<td>21.3.1996</td>
<td>O11</td>
<td>HUII HLIi A2 TR3 R2</td>
<td>0.89</td>
<td>Ct</td>
<td>Ct</td>
<td>A Fz N S</td>
</tr>
<tr>
<td>AS66</td>
<td>21.3.1996</td>
<td>O11</td>
<td>HUII HLIi A2 TR3 R2</td>
<td>1.15</td>
<td>Ct</td>
<td>Ct</td>
<td>A Fz N S</td>
</tr>
<tr>
<td>AS67</td>
<td>25.3.1996</td>
<td>OUT</td>
<td>HUII HLIi A2 TR3 R2</td>
<td>1.20</td>
<td>Ct</td>
<td>Ct</td>
<td>A Fz N S</td>
</tr>
<tr>
<td>AS68</td>
<td>29.3.1996</td>
<td>OUT</td>
<td>HUII HLIi A2 TR3 R2</td>
<td>1.04</td>
<td>Ct</td>
<td>Ct</td>
<td>A Fz N S</td>
</tr>
</tbody>
</table>

Abbreviations: —, partial digestion; UP, unique pattern; Ct, cytotoxic; Cr, cell rounding; A, ampicillin; C, chloramphenicol, Co, co-trimoxazole; Cf, ciprofloxacin; Fz, furazolidone; N, neomycin; Na, nalidixic acid; Nx, norfloxacín; S, streptomycin; ND, Not done.

![Figure 2](image)
caused by O10 in East Delhi, India (26), and an epidemic
O10 and O12 in February 1994 in Lima, Peru (7), another
literature. These include an outbreak caused by
O1, non-O139 serogroups have been described in the recent
At least three localized outbreaks of diarrhea caused by non-
important role in the evolution of toxigenic
finding that environmental nontoxigenic, non-O1 strains play
precipitate a cholera-like syndrome and the capacity to flare
strains of non-O1, non-O139
V. cholerae
V. cholerae
V. cholerae
V. cholerae
V. cholerae
V. cholerae
V. cholerae
V. cholerae
V. cholerae
V. cholerae
V. cholerae
V. cholerae
V. cholerae
V. cholerae
V. cholerae
V. cholerae
V. cholerae
V. cholerae
V. cholerae
V. cholerae
FIG. 3. Ribotypes of non-O1, non-O139 V. cholerae strains. Lanes 1 and 2, strains AM107 and AS61 (strains showing the R1 pattern), respectively; lanes 3 to 5, strains AS66, AS67, and AS68 (strains showing the R2 pattern), respectively; lanes 6, strain AM109 (a strain showing the R3 pattern); lane 7, strain AM111 (a strain showing the R4 pattern); lane 8, strain AM112 (a strain showing the R5 pattern); lane 9, strain AM113 (a strain showing the R6 pattern). The positions of bacteriophage λ HindIII molecular size markers are indicated by bars from top to bottom (23.13, 9.41, 6.55, 4.36, 2.32, and 2.0 kb). The patterns for only representative strains are shown.
the profiles obtained by ribotyping and serogrouping. For example, all O144 strains had identical patterns (Fig. 4, lanes 11, 12, and 13). Likewise, strains belonging to O11 and OUT also displayed nearly similar profiles (Fig. 4, lanes 6 to 10). The PFGE profiles of strains belonging to serogroups O144 and O11 and OUT differed from each other and from the patterns displayed by the reference strains belonging to toxigenic V. cholerae serogroups O1, O139 and those belonging to other non-O1, non-O139 serogroups. The genomic restriction patterns of individual strains were compared quantitatively, and the percent similarity in restriction patterns between the strains was estimated and is represented as a dendrogram (Fig. 5). It can be seen from the dendrogram that strains belonging to serogroups O144, O11, and OUT exhibited similar restriction patterns to form two different clusters and showed a 36.8% similarity to each other. The other interesting finding was that the O144 cluster and the O11 and OUT cluster were more closely related to the toxigenic V. cholerae O1/O139. However, all the non-O1, non-O139 serogroups from clinical sources possessing the tcpA gene, for example, by horizontal transfer, they could acquire the CTX element through exposure to the toxinoferous phage CTXφ (34). However, given the rare occurrence of strains of the non-O1, non-O139 serogroups from clinical sources possessing the tcpA and the CTX genetic elements (18), it would appear that when and if such an event occurs there would possibly be attendant changes in the somatic antigen. Molecular biology-based studies have provided evidence of the horizontal transfer of the O antigen, since isolates with nearly identical astd gene sequences had different O antigens and isolates with the O1 antigen did not cluster together but were found in different lineages (15).

FIG. 4. PFGE profiles of non-O1, non-O139 V. cholerae strains obtained with the NotI enzyme. Lanes 1 and 18, bacteriophage λ ladder; lane 2, SG08 O1 classical Inaba; lane 3, V5 O1 El Tor Ogawa; lane 4, SG26 (O139); lane 5, V. fluvialis AS63; lane 6, AS64 (O11); lane 7, AS65 (O11); lane 8, AS66 (O11); lane 9, AS67 (OUT); lane 10, AS68 (OUT); lane 11, AS60 (O144); lane 12, AS61 (O144); lane 13, AM107 (O144); lane 14, AM111 (O12); lane 15, SG2 (O60); lane 16, SG9 (O37); lane 17, SG12 (O2).

DISCUSSION
The mechanism of pathogenesis and the factors involved in the virulence of non-O1, non-O139 V. cholerae remain enigmas. It is now clear beyond a reasonable doubt that some strains of non-O1, non-O139 V. cholerae have the capacity to precipitate a cholera-like syndrome and the capacity to flare into a localized outbreak. There has been an escalation in interest in the non-O1, non-O139 serogroups following the discovery of the O139 serogroup (1, 24) and following the finding that environmental nontoxigenic, non-O1 strains play an important role in the evolution of toxigenic V. cholerae (15). At least three localized outbreaks of diarrhea caused by non-O1, non-O139 serogroups have been described in the recent literature. These include an outbreak caused by V. cholerae O10 and O12 in February 1994 in Lima, Peru (7), another caused by O10 in East Delhi, India (26), and an epidemic caused by non-O1 V. cholerae that produced ST among Khmers in a camp in Thailand (3).

The strains of non-O1, non-O139 V. cholerae characterized in this study belonged to different O serogroups and lacked all the known virulence traits associated with toxigenic V. cholerae O1, O139 and those associated with toxigenic E. coli. In particular, all the non-O1, non-O139 strains lacked the genes comprising the core part of the CTX genetic element and also the tcpA gene, both of which are recognized as important components contributing to the pathogenicity of toxigenic V. cholerae O1, O139. However, all the non-O1, non-O139 strains examined in this study possessed the gene encoding the regulatory protein ToxR, which controls the coordinate expression of genes associated with pathogenicity in toxigenic V. cholerae O1/O139. In addition, all the strains also possessed the 17-bp target sequence termed attRS1.

In this context, the most significant finding from the PFGE study was that strains of V. cholerae belonging to serogroups O144, O11, and OUT were more closely related to the toxigenic V. cholerae O1/O139 strains than to strains of non-O1, non-O139 V. cholerae associated with sporadic infections. The fact that the non-O1, non-O139 strains isolated in this study possess the basic regulatory and insertional elements necessary to receive the CTX genetic element coupled with the proximity of some of these serogroups to the O1, O139 cluster in the dendrogram suggests that the non-O1, non-O139 serogroups could be a proto-cholera agent. If these non-O1, non-O139 serogroups were to acquire the TCP gene, for example, by horizontal transfer, they could acquire the CTX element through exposure to the toxinoferous phage CTXφ (34). However, given the rare occurrence of strains of the non-O1, non-O139 serogroups from clinical sources possessing the tcpA gene and the CTX genetic elements (18), it would appear that when and if such an event occurs there would possibly be attendant changes in the somatic antigen. Molecular biology-based studies have provided evidence of the horizontal transfer of the O antigen, since isolates with nearly identical astd gene sequences had different O antigens and isolates with the O1 antigen did not cluster together but were found in different lineages (15).
In the absence of CT and TCP proteins, what is clear from this study is that some non-O1, non-O139 strains precipitate diarrhea by a mechanism which is entirely different from that used by toxigenic *V. cholerae* O1, O139. Studies with the O10 and O12 strains of *V. cholerae* isolated from the outbreak in Peru showed that none of the strains produced enterotoxin but the majority of the strains produced a cytotoxin, as assessed in Y1 and HeLa cells (7). Interestingly, all the strains of *V. cholerae* isolated in the study also produced a cytotoxin, while all strains examined produced NMDCY. We have previously reported that the purified NMDCY has impressive enterotoxic activity (28) and is widely distributed among strains of *V. cholerae*, irrespective of serogroup (27). Studies on the clonality of non-O1, non-O139 *V. cholerae* strains by RFLP analysis of rRNA genes and of the other genes examined and by PFGE collectively indicate that the upsurge which occurred in February and March 1996 was caused by strains belonging to different clones. Overall there was an excellent correlation between the results of ribotyping, RFLP analysis of various genes, and PFGE, with strains belonging to a particular serogroup (e.g., O144, O11, and OUT) showing nearly identical restriction patterns and PFGE profiles. This would indicate that a discrete set of *V. cholerae* serogroups had, for reasons that are unclear, a competitive advantage at that point in time.

Although controversial, the El Tor hemolysin has been implicated as a virulence factor for toxigenic *V. cholerae*. All the strains of *V. cholerae* examined in this study had the genetic potential to produce the El Tor hemolysin and the novel hemolysin recently described by Nagamune et al. (19). The El Tor hemolysin is reportedly a potent toxin with both enterotoxic and cytotoxic activities (2, 11, 12). The *V. cholerae* O10 and O12 strains associated with the outbreak in Peru were all positive for the El Tor hemolysin, cytotoxin, invasiveness, and mannosensitive hemagglutinin, leading Dalsgaard et al. (7) to conclude that a combination of these factors and perhaps an unknown factor may have caused the diarrhea.

The pathogenic mechanism of non-O1, non-O139 *V. chol-
erae in a way resembles that of enteropathogenic E. coli which is known to be a complex and multifaceted process that manifests as severe secretory diarrhea and which is caused by antigenically similar populations (35). Among non-O1, non-O139 V. cholerae strains, some serogroups (e.g., O10, O11, O12, and O144) seem to be more often associated with disease, despite the absence of the virulence package (ctxA, ctxB, zot, ace, and other genes), indicating that these serogroups have a mode of pathogenesis different from that of toxigenic V. cholerae. The inability to identify such serogroups (clones) earlier was related to the fact that serotyping of non-O1, non-O139 V. cholerae was not systematically pursued, and therefore, some pathogenic clones remained unidentified. There have been earlier instances in which clones of non-O1 V. cholerae have caused fairly large outbreaks, like the one caused by non-O1 V. cholerae in the Kumbh Fair at Allahabad in India in early 1954 (10). Likewise, the predilection of a serogroup was also observed among people with infections caused by non-O1 V. cholerae in Cancun, Mexico, with Smith serotype 12 accounting for 46% of the infections (8). Therefore, as for enteropathogenic E. coli, we would like to propose the nomenclature of enteropathogenic V. cholerae (EPVC) to include serogroups of non-O1, non-O139 V. cholerae that do not possess the CTX genetic element but that can cause diarrhea in humans by a hitherto unknown mechanism. It must be emphasized that at present the proposal of EPVC is based on data for a relatively small number of patients. Apart from serotyping, there are no other good markers to identify such clones of non-O1, non-O139 V. cholerae associated with diarrhea compared to their innocuous environmental counterparts. On the basis of the comparison of the pad gene sequences of a diverse collection of V. cholerae, Karaisis et al. (15) opined that it is also probable that some O antigens of V. cholerae are compatible with pathogenesis. As a beginning, isolates of serogroups such as O1, O139 V. cholerae were identified among human pathogens in an estuary. Ph.D. thesis. University of Maryland, College Park.


Takeda, T., Personal communication.


Torneiporth, N. G., J. John, K. Salgado, P. D. Jesus, E. Latham, M. C. N.,
