

## Short Report: Environmental *Vibrio Cholerae* O139 May Be the Progenitor of Outbreak of Cholera in Coastal Area of Orissa, Eastern India, 2000: Molecular Evidence

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Cholera has been reported in the state of Orissa, India during the past decades. An outbreak of diarrheal disease occurred during November 1 to November 9, 2000 in Rusipada village near Puri, which was inhabited by a population of approximately 560. During the outbreak, *Vibrio cholerae* O139 strains were isolated from clinical specimens collected from patients with acute diarrhea admitted to the infectious diseases (ID) hospital in Puri and from environmental samples collected from multiple bodies of water in the village. The index case with acute diarrhea was a 60-year-old female resident of Rusipada who had not visited any known outbreak-related areas, including an outbreak 1 week prior to her symptom onset in a nearby village. All the isolated strains were positive for *ctxA*, *tcpA*, *ace*, and *zot* genes, produced cholera toxin, and exhibited a similar antibiogram pattern. Comparison of DNA fingerprinting analysis by randomly amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE) method and dendrogram constructed from RAPD revealed that genetic homogeneity exist between the clinical and environmental O139 strains.

Epidemic and endemic cholera is a major public health problem in many developing countries and continues to be an important cause of morbidity in many areas of Asia, Africa, and Latin America. Among more than 200 serogroups of *V. cholerae* so far identified,<sup>1</sup> only O1 and recently identified O139 serogroup<sup>2–5</sup> are capable of causing epidemic cholera. It is now widely accepted that O139 Bengal like O1 and non-O1 and non-O139 *Vibrio* may survive better in aquatic environments<sup>6–8</sup> and environmental water is the reservoir for infectious *V. cholerae*.<sup>7,9</sup> In this report we investigated the clonal relationship between strains of *V. cholerae* collected both from stool specimens from persons with acute diarrhea and from environmental sources of water. During the outbreak of diarrhea, 23 non-randomly selected rectal swabs were collected from the hospitalized diarrhea patients in ID hospital, Puri in Cary-Blair Transport (CBT, DIFCO, USA) medium and transported to the Microbiology Department of Regional Medical Research Centre (RMRC), Bhubaneswar and bacteriologically analyzed following standard technique.<sup>10</sup> Subsequently after the isolation of *V. cholerae* O139 as the etiological agent of the outbreak, 20 water samples non-randomly selected were collected from water bodies situated at various distances in that village to determine the source of contamination following a previous method.<sup>11</sup> Presumptive identification of 10 and 6 *V. cholerae* were isolated from rectal swabs and water samples respectively and the strains were agglutinated with monoclonal O139 antiserum supplied by NICED,

Kolkata, India and were confirmed to belong to *V. cholerae* serogroup O139.

To investigate the similarities of clinical and environmental strains of *V. cholerae* O139, drug susceptibility test, cholera toxin assay, detection of virulent genes by polymerase chain reaction (PCR) assay, RAPD finger printing assay, and PFGE were performed.

A monosialoganglioside (GM<sub>1</sub>) enzyme-linked immunosorbent assay (ELISA) was used to examine cholera toxin production in *V. cholerae* O139 strains by the method Svennerholm and Holmgren.<sup>12</sup> Drug susceptibility test was performed following the method described elsewhere,<sup>13</sup> with the antibiotics (Hi-media Laboratories, Bombay, India) ampicillin (A, 10 µg), chloramphenicol (C, 30 µg), co-trimoxazole (Co, 25 µg), ciprofloxacin (Cf, 5 µg), furazolidone (fz, 100 µg), gentamicin (G, 10 µg), neomycin (N, 30 µg), nalidixic acid (Na, 30 µg), norfloxacin (Nx, 10 µg), streptomycin (S, 10 µg) and tetracycline (T, 30 µg). Characterization of strains as susceptible or resistant was based on size of the inhibition zone around each disc according to manufacturer's instructions, which matched interpretive criteria recommended by the WHO.<sup>14</sup> 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*.<sup>15</sup>

The presence of virulent genes *ctxA*, *tcpA*, *zot*, and *ace* were determined by using PCR assay as described elsewhere.<sup>16–18</sup> The primers used for this assay and the expected amplicon sizes are listed in Table 1. *V. cholerae* O1 serotype Inaba biotype classical strain 569B, *V. cholerae* O1 serotype Ogawa biotype El Tor strain 20(VC20) and O139 isolated in 1992 in Calcutta (SG24) were used as PCR positive control for *ctxA*, *tcpA*, *zot*, and *ace* genes. RAPD was carried out following the method described previously.<sup>19,20</sup> The pictures obtained from RAPD (1281) fingerprinting analysis of clinical and environmental strains O139 were compared with ascertaining the phylogenetic relationship among them by using Quantity-1 software.

Genomic DNA of *V. cholerae* O139 was prepared in agarose plugs as described previously to carry out PFGE.<sup>11</sup> PFGE was performed using the counter clamped homogeneous electric field method on a CHEF Mapper system (Bio-Rad, CA) with 1% PFGE grade agarose in 0.5X TBE buffer. Run condition was generated by auto algorithm mode of CHEF Mapper, PFGE system using a size range of 20–300 kb marker for *V. cholerae* strains. The gel was stained in 10 µg/mL ethidium bromide solution for 30 minutes, de-stained in water for 15 minutes, and photographed under UV light in Alpha Imager (Alpha Infotech Corporation, USA).

During 9 days of outbreak, 198 persons had acute diarrhea (1–2 members from each family). Members of the affected family came into close physical contact while handling the patients. Investigations were initiated immediately after the

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TABLE 1  
PCR Primer sequences used for the detections of genes specific for *V. cholerae*

Gene	Primer sequence	Amplicon size (bp)	Reference
<i>ctxA</i>	F-CTCAGACGGGATTTGTTAGGCACG R-TCTATCTCTGTAGCCCCTATTACG	301	16
<i>tcpA</i> (El Tor)	F-GAAGAGTTTGTAAGAAGAACAAC R-GAAGGACCTTCTTTACGTTG	471	16
<i>Zot</i>	F-TCGCTAACGATGGCGCGTTTT R-AACCCCGTTTCACTTCTACCCA	947	17
<i>ace</i>	F-TAAGGATGTGCTTATGATGGACACCC R-CGTGATGAAATAAAGATACTCATAGG	316	18
1281	AACGCGCAAC		19, 20
1283	GCGATCCCA		19, 20

onset of the outbreak and necessary measures were taken by local health authorities to control the outbreak.

Immediate steps were taken on November 2, 2000 to examine the water from different water bodies in the village. This village has 80 households located 6 kilometers away from the Bay of Bengal. Of the total number of households, 78 families use 4 shallow wells, 3 common ponds, 14 individual ponds, and 23 tube wells; 2 families use individual ponds located on their premises. A small canal carrying water from Mahanadi river (the main river of the state) passes adjacent the village. Except for a few households that have an individual latrine, most of the people defecate in the open around the water bodies. Seventy eight families affected by diarrhea used common and individual ponds for domestic use, bathing (humans and domestic animals), and washing utensils. Two unaffected family members do not use a common pond for any purposes and drink water from their personal well. Open wells and tube wells are used for drinking purposes. Due to hard water coming in the tube well and shallow well, people more commonly use the pond water for cooking and some-

times for drinking. All the environmental *V. cholerae* O139 were isolated from the common ponds used by the affected families. In the previous week an outbreak of diarrhea occurred in a neighboring village, which may have been due to the use of pond water in a marriage ceremony. Rusipada residents attended this ceremony.

*V. cholerae* O139 strains isolated from patients and surface water revealed a similar drug resistance pattern. Strains were resistant to AFRNSNa and sensitive to CfNxCTGCo. We report for the first time that the isolation of nalidixic acid-resistant *V. cholerae* O139 was the most remarkable feature.

PCR analysis of all the *V. cholerae* O139 strains revealed a positive result for the *ctxA*, *tcpA*, *zot*, and *ace* genes. The cholera toxin concentration produced by patient and environmental O139 was almost equal varying between 200 and 400 pg/ml. The *NotI* cleavage pattern of the DNA of strains from patient and environment were identical (Figure 1). RAPD analysis of *V. cholerae* O139 strains showed an identical PCR fingerprinting profile ranging from 300 bp to 2 kb (Figure 2A—1281 and 2B—1283). The dendrogram constructed from the RAPD fingerprinting of O139 strains of (Figure 2A—1281) form one cluster (Figure 3).

The experience at Rusipada village presumes that the movement of people between the two villages may have played a role in transmission and spread of the outbreak by contaminating water sources. Six water sources (common ponds) could be identified for the presence of *V. cholerae* O139.

Complete phenotypic and genotypic characterization of the

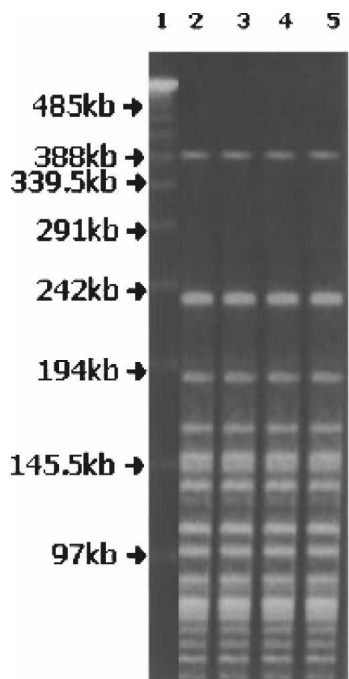


FIGURE 1. PFGE profile generated with *NotI* digested genomic DNA of *V. cholerae* O139 strains. Lane 1: Bacteriophage  $\lambda$ - ladder; Lane 2 & 3: O139, clinical; Lane 4 & 5: O139; environmental.

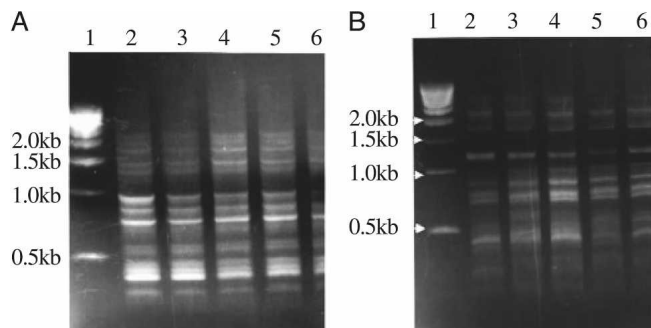


FIGURE 2. (A) and (B) depict comparison of the RAPD profiles of *V. cholerae* O139 strains from environmental and clinical sources of the same locality using primer 1281 & 1283 respectively. In each figure Lane 1: 500 bp ladder; Lane 2–4: (WRD2, WRD4 & WRD5) environmental *V. cholerae* O139; Lane 5 & 6: (Pocy 192 & Pocy 194) clinical *V. cholerae* O139.

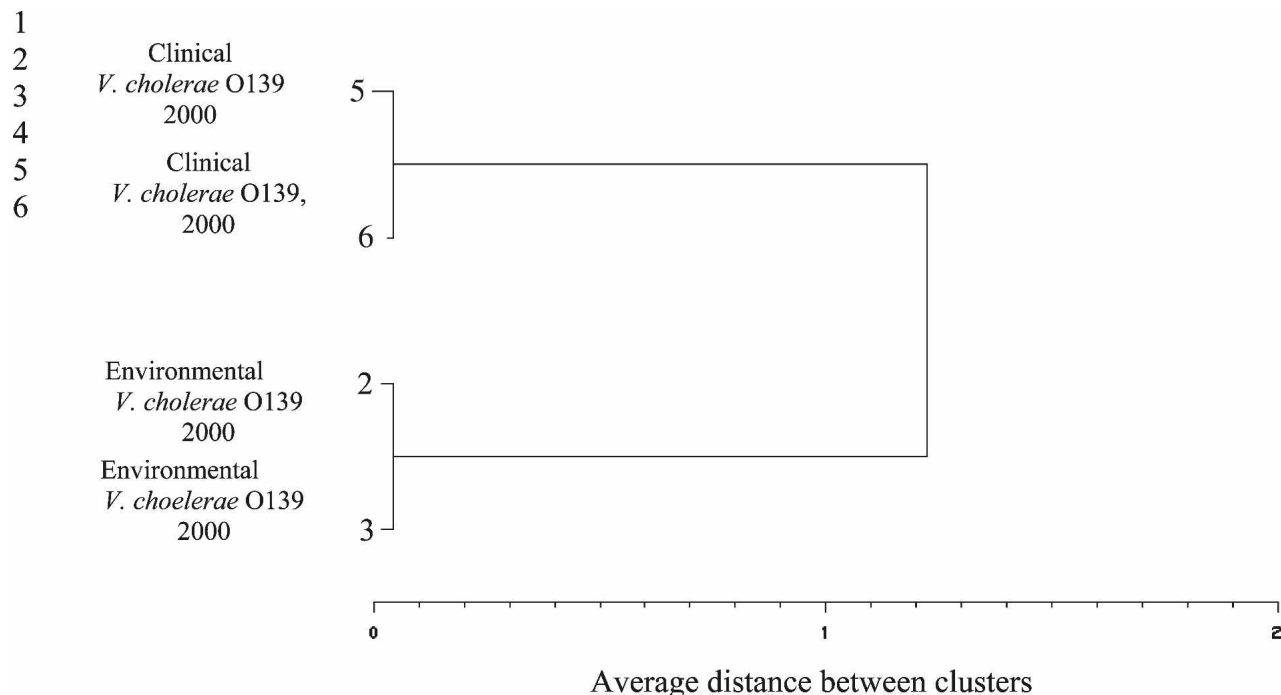


FIGURE 3. Dendrogram (UPGMA) of clinical and environmental strains based on average linkage cluster analysis using RAPD primer (1281).

clinical and environmental strains of O139 was done to determine the genetic relatedness. In the present study, equal amount of in vitro synthesis of CT and similar antibiotic susceptibility pattern of patient and environment O139 strains showed homologous phenotypic characters, which was in effect and was confirmed by RAPD and PFGE. In vitro synthesis of cholera toxin by environmental O139 strains hypothesized that these strains can adhere, colonize, and produce CT in small intestine and have all the characteristics of epidemic cholera.<sup>21</sup> Because environmental O139 strains in this study had the ability for in vitro synthesis of CT, these can be suggested as the epidemic strains that reemerge from the environment after inter-epidemic period. Again both strains had similar phenotypic and genotypic character and belonged to one cluster. As *V. cholerae* O139 strains that caused epidemic and O139 present in the environment possessed similar phenotypic and genotypic character belonging to one cluster and had the ability to produce CT, these observations therefore hypothesize that environmental O139 strains may have been the progenitor of the outbreak strains. Another possible explanation for this outbreak is that environmental isolates of *V. cholerae* may have been due to fecal contamination of water sources before these were sampled and tested. This is the first report of isolation of O139 from the environment, which might be the cause of a sudden outbreak of cholera in this geographical region. Further ecological study is required to understand the inter-phase between the host and environment, toxigenic status, and fluctuation in the antibiotic resistance pattern.

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