Original Article

Sequential outbreaks due to mixed ctxB alleles of Vibrio cholerae O1 in Mayurbhanj district of Odisha, India

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Abstract

Introduction: Cholera is a significant threat causing outbreaks/epidemics with high morbidity and mortality in coastal and tribal districts of Odisha. A sequential cholera outbreak reported from four places in Mayurbhanj district of Odisha during June to July 2009 was investigated.

Methodology: Rectal swabs from diarrhea patients were analyzed for the identification, antibiogram profiles and detection of ctxB genotypes by double mismatch amplification mutation (DMAMA) polymerase chain reaction (PCR) assays and sequenced. The different virulent and drug resistant genes were detected by multiplex PCR assays. The clonality analysis on selected strains was done by pulse field gel electrophoresis (PFGE).

Results: Bacteriological analysis of rectal swabs revealed the presence of V. cholerae O1 Ogawa biotype El Tor which were resistant to co-trimoxazole, chloramphenicol, streptomycin, nalidixic acid, erythromycin, furazolidone and polymyxin B. DMAMA-PCR assay revealed that the cholera outbreak in Mayurbhanj district was due to both ctxB1 and ctxB7 alleles of V. cholerae O1 El Tor strains. All the V. cholerae O1 strains were positive for all virulence genes. The multiplex PCR assay on V. cholerae O1 strains revealed the presence of antibiotic resistance genes like dfrA1 (100%), intSXT (100%), sulII (62.5%) and StrB (62.5%). PFGE results on V. cholerae O1 strains exhibited two different pulsotypes with 92% similarity.

Conclusions: This outbreak was a transition phase where both ctxB genotypes were prevalent after which the ctxB7 genotype gradually became dominant in Odisha. Therefore, close monitoring and continuous surveillance on diarrheal disorders is essential to prevent the future diarrheal outbreaks in this region.

Key words: V. cholerae O1, ctxB1, ctxB7, cholera outbreak, Mayurbhanj.


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Introduction

Cholera is caused by Vibrio cholerae and is reported throughout the world, predominantly in the developing and underdeveloped countries of South Asia, Africa and Latin America [1,2]. V. cholerae has more than 200 serogroups based on the somatic O-antigens. Among the serogroups, V. cholerae O1 and O139 serogroups are mainly responsible for outbreaks and epidemics [3]. V. cholerae O1 is further divided into classical and El Tor biotypes on the basis of phenotypic and genotypic differences. The classical biotype is believed to be extinct after the 6th pandemic, whereas the El Tor biotype continues to circulate until now as the 7th pandemic strain of cholera. The El Tor biotype of V. cholerae has undergone rapid genetic modification and recombination to form different variants over time which ranged from the ctxB1 to ctxB9 [4,5]. Following the cholera outbreak in Haiti in 2010, cholera outbreaks in recent years around the world have been attributed to the El Tor variant of V. cholerae O1.

The El Tor variant of V. cholerae O1 strains have been spreading the cholera epidemic after the Haiti epidemic during 2010 and were predominant in South Asia and Bangladesh [6,7]. Outbreaks were caused by these variants in Nepal in 2012, Eastern Africa (Tanzania, Kenya and Uganda) in 2015 and more recently in Yemen in 2016-2017 [8]. Similarly, the El Tor variant of V. cholerae O1 was isolated in the Indian states of West Bengal in 2006 [9], Punjab and Haryana in 2007 [10], as well as in Odisha in 1999 [11].

In 2007, the El Tor variants of V. cholerae O1 caused severe cholera outbreaks and epidemics in three tribal districts of Odisha, India: in the Rajnagar block of Kendrapada district in 2009 [12], in Balasore and Rayagada districts in 2016 [13] and in Rayagada and Baragarh districts of Odisha in 2018-2019 [14]. The present study investigated the causative agent of the sequential diarrheal outbreaks, the antibiogram profile and detection of various toxic genes of V. cholerae and their clonality reported in three different villages
(Badkuldiha, Pandra, Sindurgaura) and Karanjia NAC of Mayurbhanj district, Odisha from June to July, 2009.

**Methodology**

**Study Area**

Based on the information received from Rangamatia primary health centre (PHC), the medical officers of the local community health centre visited the diarrhea affected villages (Sindurgaura, Badkuldiha and Pandra) and Karanjia NAC of Mayurbhanj district during the months of June and July 2009. Information was collected about the index case, date-wise line listing of all diarrhea cases, deaths, and clinical signs and symptoms of the patients. The sources and chlorination of drinking water and prevailing sanitary conditions in all villages were also recorded.

**Isolation and Identification**

Rectal swab samples from the diarrhea patients of the affected villages and hospitals were collected before administration of antibiotics by the Rangamatia PHC staff and sent to the Indian Council of Medical Research (ICMR)-Regional Medical Research Centre, Bhubaneswar in Cary-Blair transport medium. The samples were put in enriched alkaline peptone water and streaked on Hektoen enteric agar, Mac Conkey agar samples were put in enriched alkaline peptone water (ICMR)-Regional Medical Research Centre, Bhubaneswar in Cary-Blair transport medium. The samples were put in enriched alkaline peptone water and streaked on Hektoen enteric agar, Mac Conkey agar and thiosulfate-citrate-bile salt-sucrose agar (TCBS) (Becton Dickinson, Sparks, USA) plates. Subsequently, sucrose fermenting colonies from TCBS agar plates were selected and accessed via selective biochemical tests. Serological confirmation was performed using V. cholerae O1 polyvalent and monovalent Ogawa and Inaba antisera (Becton Dickinson, Sparks, USA). The isolation and identification of the pathogens were conducted according to our earlier protocols adopted in the laboratory, in accordance with the World Health Organization (WHO) guidelines [11].

**Antibiotic susceptibility assay**

The resistance and sensitivity patterns of V. cholerae O1 strains were assessed using commercial antibiotic discs (Becton Dickinson, Sparks, USA based on our earlier protocols adopted in the laboratory [15]. The antibiotics used were ampicillin (A, 10 µg), cotrimoxazole (Co, 25 µg), Chloramphenicol (C, 30 µg), ciprofloxacin (Cip, 5 µg), furazolidone (Fr, 50 µg), gentamicin (G, 10 µg), neomycin (N, 30 µg), norfloxacin (Nx, 10 µg), nalidixic acid (Na, 30 µg), streptomycin (S, 10 µg), erythromycin (E, 15 µg), tetracycline (T, 30 µg), azithromycin (Azm, 15 µg), ofloxacin (Ofl, 5 µg), doxycycline (Do, 30 µg) and polymyxin B (Pb, 50U) (Becton Dickinson, Sparks, USA). The resistance profiles of the isolates were determined by measuring the zone of inhibition and comparing it within an interpretative chart to determine the sensitivity of the antibiotics based on the specific chart supplied by the manufacturer.

**Double Mismatch Amplification Mutation (DMAMA) polymerase chain reaction (PCR) Assay**

DMAMA PCR assay was performed to detect the types of ctxB allele in all V. cholerae O1 strains isolated from all outbreak villages of Mayurbhanj district as mentioned above. The PCR assay was performed using the primer set ctxB F4/Rv Cla and ctxB3/ Rv Cla for the Haitian and Classical ctxB allele respectively following the protocols described by Naha et al. [9].

**Sequence**

The ctxB gene of some selected V. cholerae O1 strains from these outbreak areas were amplified using primers ctxB F/R [9]. The gel-purified products were directly sequenced and compared with the sequences of V. cholerae O1 control strain O395 (Classical) and N18691 (El Tor) with accession no. NC012582 and AE003852 respectively from Gen Bank database and multiple sequence alignments were performed using Clustal W of Mega 6.0 (Gen Bank accession no. MK720613 and MK720614).

**tcpA gene analysis**

PCR amplification of tcpA gene was carried out on some selected V. cholerae O1 strains from these outbreak areas to determine whether the V. cholerae O1 strains were Classical, El Tor or Haitian alleles of the tcpA gene. The PCR assay conditions and primers were based on Ghosh et al. [16].

**Analysis of virulence genes**

All V. cholerae O1 strains from these outbreak areas were studied for the detection of virulence, serogroups and toxigenic genes through two separate multiplex PCR (mPCR) assays. The first mPCR (mPCR I) assay was used to confirm the presence of V. cholerae serogroup and its toxigenicity by using four sets of primers for genes encoding the outer membrane protein (ompW), somatic antigen (rfbO1), cholera toxin CT (ctxA) and toxin regulated pilus (tcpA). The second mPCR (mPCR II) was carried out to confirm the presence of the virulence genes encoding the accessory cholera enterotoxin (ace), repeat in toxin protein (rtxC), outer membrane protein (ompU) and toxin regulator (toxR). The PCR assay conditions and primers used for
both the mPCR assays were followed by the method as described by Kumar et al. [17].

Assay for antibiotic resistant genes

The presence of antibiotic resistant genes of some selected *V. cholerae* O1 strains from three outbreak areas encoding for sulfamethoxazole (*SulII*), trimethoprim (*dfrA1*), streptomycin (*StrB*) and the *SXT* genetic element were determined by using multiplex PCR assay. The multiplex PCR assay was carried out by using four primer pairs of *sulII*, *dfrA1*, *strB* and *SXT* simultaneously in the same reaction mixture. The PCR primers and the assay conditions were performed based on Ramachandran et al. [18].

Pulse field gel electrophoresis (PFGE)

The PFGE was conducted to type some selected *V. cholerae* O1 strains isolated from the present outbreak areas following the standardized pulsenet protocol [19]. Electrophoresis was performed with pulse time ranging from 2 to 10 s for 13h and from 20 to 25 s for 6 h at 6 V on CHEF MAPPER (Bio Rad, Hercules, USA). The gel was stained with ethidium bromide and photographed in a gel documentation unit (Bio Rad, Hercules, USA). PFGE patterns were examined with Bionumerics software version 6.2 (Applied Maths NV, Keistraat, Belgium) and the dendrogram analysis was done based on the interpretation criteria described by Tenover et al. [20].

Results

Diarrhea Outbreak

All the three diarrhea affected villages (Badkuldiha, Sindurgaura and Pandra) and Karanjia NAC are located in the Mayurbhanj district of Odisha state in India (Figure 1). The index case of Sindurgaura village was that of a 55 years old female who suffered from profuse rice watery stool, vomiting, abdominal cramping, and vomiting on 18 June 2009 after consuming water from the nearby open well water and developed severe dehydration with collapsing state and died on morning of 19 June 2009. There were 39 wells and 8 tube wells located in that village. The people were using water from the open wells for bathing, washing and cooking during events and festivals. The village had a population of 1667, of which 13 diarrhea patients were admitted to Rangamati PHC between 18 and 23 June 2009. The incidence rate was 7.79% and case fatality rate was 7.6% (Figures 2 and 3).

Similarly, another incidence of diarrhea case was reported in Badkuldiha village of Rangamati block. One 30 years old male went to attend a funeral ceremony of a person deceased following a diarrhea case in Sindurgaura village and returned to his own village (Badkuldiha) on 23 June 2009 morning. After attending the funeral ceremony, he suffered from profuse watery diarrhea with rice watery stool and vomiting, and was admitted to the District Headquarter Hospital (DHH) in Baripada. He bathed and cleaned his diarrhea-infected clothes near a well. The fecal materials from the feces along with vomitus might have mixed with the open well water. Those families who used that well water for drinking and household purposes reported diarrhea on 24 June 2009, possibly infected through the well water. The Badkuldiha village had 165 households with a total of 785 people. There were 18 wells and 5 tube wells located in that village. Fifteen diarrhea patients (24 to 26 June 2009) were admitted to the nearest health center. Overall, the incidence rate was 19.10 %, with no death (Figures 2 and 3).

The index case of Pandra village of Rangamati block was a 40 years old male who suffered from loose motion, vomiting and abdominal pain on 13 July 2009 after consuming water from the open well. The village had a population of 1721 with 500 households. The number of severe diarrhea cases increased up to 37, including one death on17 July 2009 (incidence rate (IR); 21.4 % and case fatality rate (CFR; 2.7%)). There were 43 open wells and 10 tube wells in that village (Figures 2 and 3).

Another diarrheal outbreak was reported on 22 July 2009 at ward no. 8 of Karanjia NAC, Mayurbhanj district. People from nearby ward no. 12 were also affected on 22 July 2009. The source of infection was suspected as well water used for drinking and household purposes. The onset of diarrhea began on 22 July 2009 and a total of 29 diarrhea cases was registered by 29 July 2009. Most of the diarrhea cases presented symptoms of rice watery stool, vomiting, abdominal cramping and severe dehydration. The total population...
of the Karanjia NAC was 7449. Twenty-nine diarrhea patients were admitted to Baripada hospital, and only one case fatality rate was 3.4% (Figures 2 and 3, Table 1).

**Identification of bacterial pathogens**

All the isolates from these three different villages and Karanjia NAC were positive for *V. cholerae* O1 Ogawa biotype El Tor. The antibiotic susceptibility test was conducted on all the isolated *V. cholerae* O1 strains. All the strains were sensitive to tetracycline, gentamicin, norfloxacin, ciprofloxacin and doxycycline. However, these strains were resistant to chloramphenicol, streptomycin, ampicillin, nalidixic acid, erythromycin, furazolidone and polymyxin B.

**V. cholerae genotyping**

All the clinical isolates of *V. cholerae* O1 from these three different villages and Karanjia NAC were subjected to DMAMA PCR assay for the ctxB genotype. It was observed that El Tor biotypes harbored with both ctxB1 (37.5%) and ctxB7 (62.5%) showing dominance of ctxB7 genotypes over ctxB1, irrespective of the location or source of isolation. No water samples were positive for *V. cholerae* O1 as high chlorination was done in the open well water before the collection of samples as reported from this outbreak (Table 2).

**Analysis of tcpA gene**

The tcpA gene specific PCR assay was carried out for all *V. cholerae* O1 Ogawa strains collected from the outbreak areas of Rangamatia block and Karanjia NAC. The PCR assays on all the strains were positive for tcpA Haitian gene. In order to further confirm the PCR results on tcpA gene, representative strains that resulted positive for Haitian tcpA gene were selected for sequencing of the entire tcpA gene (Table 2). The entire tcpA gene sequence of all *V. cholerae* O1 strains were deposited in GenBank database under the Gen Bank accession numbers MW887547-MW887549.

**Analysis of antibiotic resistance genes**

All *V. cholerae* O1 strains from the cholera outbreak areas were examined for the presence of different antibiotic resistance genes and the SXT element. Out of the 8 strains of *V. cholerae* O1, 5 strains produced positive results with PCR assay and amplified a 626 bp fragment of sulII, a 278bp fragment of dfrA1, a 515 bp fragment of StrB and a 1035 bp fragment of the SXT element. This correlates with resistance to

![Figure 2](image1.png)

**Figure 2.** Date wise incidence of diarrhea cases in the three villages (Sindurgaura, Badkuldiha and Pandra) and Karanjia NAC of Mayurbhanj district: June to July-2009.

![Figure 3](image2.png)

**Figure 3.** Age and gender-wise distribution of diarrhea cases in the three villages (Sindurgaura, Badkuldiha and Pandra) and Karanjia NAC of Mayurbhanj district: June to July-2009.

Table 1. Details of diarrhea cases in three villages and Karanjia NAC.

<table>
<thead>
<tr>
<th>Villages/NAC</th>
<th>Date of onset</th>
<th>Last case</th>
<th>No. of cases (IR)</th>
<th>Death rate (CFR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sindurgaura</td>
<td>18.6.09</td>
<td>23.6.09</td>
<td>13 (7.79)</td>
<td>7.6%</td>
</tr>
<tr>
<td>Badkuldiha</td>
<td>24.6.09</td>
<td>25.6.09</td>
<td>15 (19.10%)</td>
<td>0</td>
</tr>
<tr>
<td>Pandra</td>
<td>13.7.09</td>
<td>17.7.09</td>
<td>37 (21.4%)</td>
<td>2.7%</td>
</tr>
<tr>
<td>Karanjia NAC</td>
<td>22.7.09</td>
<td>29.7.09</td>
<td>29 (3.89%)</td>
<td>3.4%</td>
</tr>
</tbody>
</table>

Table 2. Distribution of ctxB, tcpA, virulent and antibiotic resistance genes of *V. cholerae* O1 isolated from cholera outbreak areas of Mayurbhanj district.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Code</th>
<th>ctxB gene</th>
<th>tcpA gene</th>
<th>ompW</th>
<th>ctxA</th>
<th>tcpO1</th>
<th>tcpA</th>
<th>ctxC</th>
<th>ompU</th>
<th>toxR</th>
<th>ace</th>
<th>Sul II</th>
<th>SXT</th>
<th>dfrA1</th>
<th>StrB</th>
<th>Antibiogram profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MY3-11</td>
<td>ctxB7</td>
<td>Haitian</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>CoSNaENFrPb</td>
</tr>
<tr>
<td>2</td>
<td>MY3-12</td>
<td>ctxB1</td>
<td>Haitian</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>CoCSNaENFrPb</td>
</tr>
<tr>
<td>3</td>
<td>MY3-13</td>
<td>ctxB1</td>
<td>Haitian</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>CoCSNaENFrPb</td>
</tr>
<tr>
<td>4</td>
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<td>ctxB7</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
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<td>ctxB7</td>
<td>Haitian</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
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<td>ctxB1</td>
<td>Haitian</td>
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<td>+</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>CoSNaFrPb</td>
</tr>
<tr>
<td>7</td>
<td>MY3-20</td>
<td>ctxB7</td>
<td>Haitian</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>CoTSGANaEFrPb</td>
</tr>
<tr>
<td>8</td>
<td>MY3-21</td>
<td>ctxB7</td>
<td>Haitian</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>CoCSNaAnFrPb</td>
</tr>
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</table>
ampicillin, furazolidone, nalidixic acid, trimethoprim, streptomycin and trimethoprim-sulfamethoxazole. Three strains of *V. cholerae* O1 produced positive results with PCR assay which amplified a 278 bp fragment of *dfrA1* and a 1035 bp fragment of *SXT* element which is responsible for the resistance to trimethoprim and other antibiotics respectively (Table 2).

**Analysis of virulence genes**

All *V. cholerae* O1 strains from all these three outbreak villages and Karanjia NAC of Rangamatia block were examined for different toxigenic and virulence genes by two separate mPCR assays. All these strains carried all the virulence and toxigenic genes (Table 2).

**Pulse field gel electrophoresis (PFGE)**

The PFGE analysis on above selected *V. cholerae* O1 strains exhibited two different pulsotypes. The ctxB7 allele of *V. cholerae* O1 strains isolated from these outbreak areas were of a single clone. However, one ctxB1 allele of *V. cholerae* O1 strain isolated from Pandara village of Rangamatia block had a different pulsotype sharing 92% similarity with ctxB7 allele of *V. cholerae* O1 strains (Figure 4).

**Discussion**

Acute diarrheal disease (cholera) is a major public health problem globally and a key indicator of lack of social development. In developing countries cholera outbreaks typically occur due to lack of access to safe drinking water and proper sanitation [21]. According to the World Health Organization (WHO) estimation, diarrhea can be reduced up to 35% when proper hygienic water and sanitation are provided [22]. The lack of safe drinking water and proper sanitation were the main cause of cholera outbreaks in developing countries and this was also observed in these villages. In the Asian region, the Indian subcontinent contributes to 78% of cholera cases. Epidemics and different outbreaks of cholera have occurred repeatedly in various places in India [22]. The availability of pure drinking water for a large portion of the Indian population is a major public health concern. Different outbreaks and major epidemics of cholera have occurred repeatedly in various locations in Odisha as well. The availability of safe drinking water and proper sanitation for a large part of the Indian population is a major public health concern which was observed from these places also [23]. Epidemics and outbreaks of acute diarrhea have frequently occurred in rural areas of tropical developing countries where drinking water is obtained from non-protected open wells, chua (sallow pit in paddy field or river bed) and bore wells which are easily contaminated by groundwater after rain and by contamination of water drawing practices from the open wells and tube wells [23]. Based on the findings in this study it is clear that the onset of the disease occurred first from Sindurgaura village and then spread to Badkuldiha, Pandra and Karanjia NAC sequentially. The Singh Sahi area of Pandra village had no tube wells. The families of Singh Sahi in Pandara village consumed water from the open wells for cooking, bathing and washing the diarrhoea contaminated clothes. This contaminated open well water served as the source of infection. Contaminated water along with poor hygiene of the households were the major source of spread of the infection. Thus, the sources of infection were contamination of open wells and tube wells, improper hygienic practices, poor environmental conditions and sanitation, and low level of awareness among the villagers.

The rectal swabs from the four outbreak areas showed mixed genotypes (ctxB1 and ctxB7) of *V. cholerae* O1 serogroup with multidrug resistance to different antibiotics. The bacteriological and DMAMA PCR analysis on all strains isolated from the four locations in the Mayurbhanj district indicated the predominance of the HCT variant (ctxB7: 62.5%) of *V. cholerae* O1 strains, whereas the diarrhea outbreak in the Kendrapara district in 2009 was due to the dominance of El Tor biotype of classical ctxB1 over Haitian ctxB7 genotypes showing resistance to ciprofloxacin, ampicillin, nalidixic acid, co-trimoxazole, streptomycin and chloramphenicol [11]. The *V. cholerae* O1 strains from the present study were resistant to ampicillin, furazolidone, nalidixic acid, streptomycin, neomycin and chloramphenicol which correlates with the earlier findings of 2007 cholera epidemic in the tribal areas of Odisha [24]. In this study, sensitivity to tetracycline, gentamicin, norfloxacin, ciprofloxacin and doxycycline was observed, whereas
sensitivity to tetracycline, norfloxacin and gentamicin was observed from the outbreak in Kendrapada district in 2009. Multiple antibiotic resistances in *V. cholerae* have emerged as a major problem worldwide [25].

Prevalence of multidrug resistant (MDR) *V. cholerae* O1 strains have increased progressively in Odisha as well as in different parts of the country since 1996 [26,27]. The rapid shift of antimicrobial resistance in *V. cholerae* O1 was not a usual phenomenon and multiple drug resistances were rare. The pattern of quick shifts in antibiotic resistance profile indicates an enhanced mobile genetic element and lateral acquisition of *intSXT* into the chromosomes which confers resistance to antibiotics [28]. Indiscriminate use of different antibiotics in different fields is resulting in MDR strains that lead to numerous challenges in antibiotic therapy worldwide. The multiplex PCR assay for the antibiotic resistance genes further confirmed the presence of antibiotic resistance genes from the present study. *intSXT* is normally associated with the *SXT* element showing resistance to many antibiotics and this was observed for all the strains in this study.

Cholera pathogenesis in *V. cholerae* is a complex process which involves the synergetic action of several genes. All the selected isolates were screened for the presence of various genes involved in the toxigenicity and pathogenicity by using two sets of mPCR. The first multiplex PCR (mPCR I) confirmed the presence of *ompW*, *ctxA*, *rfbO1* and *tcpA* genes, while the second multiplex PCR (mPCR II) analysis revealed that all the isolates were positive for *rtxC*, *ompU*, *ace* and *toxR* genes. The presence of *rfbO1* gene confirmed the serogroup O1 of all the outbreak strains. The presence of *ompW* gene confirmed that all these strains collected from outbreak villages were *V. cholerae* O1. The presence of *toxR* gene detects the cholera toxin (CT) as the most important marker among various toxin produced by *V. cholerae* and was observed in this study [1]. The RTX toxin which represents important virulence factors are widely used to discriminate the Gram-negative bacteria from others [29]. The presence of RTX toxin gene in *V. cholerae* encodes the presumptive cytotoxin (*rtxA*), an acetyltransferase (*rtxC*) and is also associated with the ATP binding cassette transporter system which is physically linked to the core element in the *V. cholerae* genome [30]. In this outbreak investigation, various other genes like *toxR* and *ace* were screened and the isolates were positive for these genes, suggesting the presence of the core toxin region in all the isolates of *V. cholerae* O1.

The PFGE analysis clearly indicated two distinct pulsotypes of *V. cholerae* O1 strains. It is assumed that the *V. cholerae* O1 strains which caused the cholera outbreak in Sindurgaura, Badkuldih and Karanjia NAC might also have been responsible for the outbreak in Pandara village and a variation occurred towards the end of this outbreak and resulted in a new pulsotype with 92% similarity. The *V. cholerae* O1 strains isolated from this outbreak proved to be highly clonal, suggestive of high genetic homogeneity in the *V. cholerae* O1 population.

**Conclusions**

Based on this study it is concluded that the sequential cholera outbreaks in 2009 that were reported in Mayurbhanj district of Odisha were due to multidrug resistant *V. cholerae* O1 Ogawa *ctxB1* and *ctxB7* (dominant) genotypes with two distinct PFGE patterns. This outbreak could also be described as a transition phase where both *ctxB1* and *ctxB7* genotypes were prevalent and after which the *ctxB7* genotypes of *Vibrio cholerae* gradually became dominant in Odisha [14]. Hence the distribution of safe drinking water and public hygiene should be of foremost importance for the control and prevention of future diarrheal outbreaks in the tribal areas of Odisha. Continuous surveillance of diarrheal outbreaks particularly due to cholera should be undertaken in the tribal areas of Odisha.

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