JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

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

Bibhuti Bhusan Pal[1], Ashish Kumar Nayak[1], Smruti Ranjan Nayak[1], Swatishree Pany[1]

[1] *Microbiology Division, ICMR-Regional Medical Research Centre, Chandrasekharpur, Bhubaneswar, Odisha, India*

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, ampicillin, 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.

J Infect Dev Ctries 2023; 17(1):73-79. doi:10.3855/jidc.16553

(Received 11 March 2022 – Accepted 26 July 2022)

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 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 adapted 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, 10µg), co-trimoxazole (Co, 25 µg), Chloramphenicol (C, 30 µg), ciprofloxacin (Cip, 5 µg), furazolidone (Fr, 50 µg), gentamicin (G, 10µg), 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 (Of, 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].

Sequencing

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)

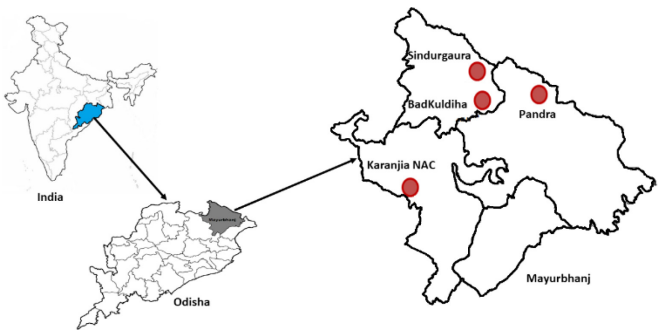
The PFGE was conducted to type some selected *V. cholerae* O1 strains isolated from the present outbreak areas following the standardized pulsenet protocol [19]. Electrophoresis was performed with pulse time ranging from 2 to 10 s for 13h and from 20 to 25 s for 6 h at 6 V on CHEF MAPPER (Bio Rad, Hercules, 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 Rangamatia PHC between 18 and 23 June 2009. The incidence rate was 7.79% and case fatality rate was 7.6% (Figures 2 and 3).

Figure 1. Map of Odisha (India) showing the severe diarrhea affected villages and Karanjia NAC in Mayurbhanj district.



Similarly, another incidence of diarrhea case was reported in Badkuldiha village of Rangamatia 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 Rangamatia 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

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

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

of the Karanjia NAC was 7449. Twenty-nine diarrhea patients were admitted to Baripada hospital, and only one death was reported. Overall, the attack rate was 3.89% and the 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 co-trimoxazole, 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. Date wise incidence of diarrhea cases in the three villages (Sindurgaura, Badkuldiha and Pandra) and Karanjia NAC of Mayurbhanj district: June to July-2009.

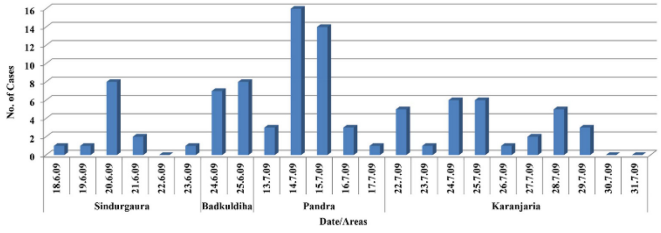


Figure 3. Age and gender-wise distribution of diarrhea cases in three the villages (Sindurgaura, Badkuldiha and Pandra) and Karanjia NAC of Mayurbhanj district: June to July-2009.

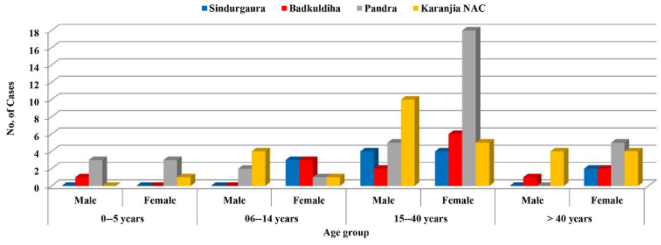


Table 2. Distribution of *ctx*B, *tcp*A, virulent and antibiotic resistance genes of *V. cholerae* O1 isolated from cholera outbreak areas of Mayurbhanj district.

Sl. No	Code	*ctx*B gene	*tcp*A gene	*omp*W	*ctx*A	*rfb*O1	*tcp*A	*rtx*C	*omp*U	*tox*R	*ace*	*Sul* II	SXT	*dfr*A1	*Str*B	Antibiogram profile
1	MYJ-11	*ctx*B7	Haitian	+	+	+	+	+	+	+	+	+	+	+	+	CoSNaEFrPb
2	MYJ-12	*ctx*B1	Haitian	+	+	+	+	+	+	+	+	-	+	+	-	CoCSANaEFrPb
3	MYJ-13	*ctx*B1	Haitian	+	+	+	+	+	+	+	+	-	+	+	-	CoSANaENFrPb
4	MYJ-16	*ctx*B7	Haitian	+	+	+	+	+	+	+	+	+	+	+	+	CoTSAtEFrPb
5	MYJ-18	*ctx*B7	Haitian	+	+	+	+	+	+	+	+	+	+	+	+	CoSNaFrPb
6	MYJ-19	*ctx*B1	Haitian	+	+	+	+	+	+	+	+	-	+	+	-	CoSNaFrPb
7	MYJ-20	*ctx*B7	Haitian	+	+	+	+	+	+	+	+	+	+	+	+	CoTCSGANaEFrPb
8	MYJ-21	*ctx*B7	Haitian	+	+	+	+	+	+	+	+	+	+	+	+	CoCSANaFrPb

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)

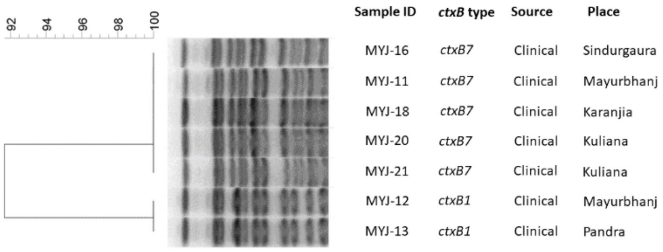
The PFGE analysis on above selected *V. cholerae* O1 strains exhibited two different pulsotypes. The *ctxB7* allele of *V. cholerae* O1 strains isolated from these outbreak areas were of a single clone. However, one *ctxB1* allele of *V. cholerae* O1 strain isolated from Pandara village of Rangamatia 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 diarrhea 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

Figure 4. Pulsed field gel electrophoresis (PFGE) analysis of V. cholerae O1 strains with different ctxB genotypes.



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, Badkuldiha 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.

Acknowledgements

We are thankful to the medical staff of the concerned PHCs and Chief District Medical Officer, Mayurbhanj for providing the samples and basic information required for this investigation. The help and co-operation rendered by the Director, Directorate of Public health, Directorate of Health services, Government. Of Odisha is acknowledged. The molecular analysis supported by the Indian Council of Medical Research, New Delhi, India (Project No. 5/8-1 (70)/2018-19- ECD-II) funding to Dr B. B. Pal is also acknowledged.

References

1. Kaper JB, Morris JG, Levine MM (1995) Cholera. Clin Microbiol Rev 8: 48-86.
2. O'Ryan M, Prado V, Pickering LK (2005) A millennium update on pediatric diarrheal illness in the developing world. Semin Pediatr Infect Dis 16: 125-136.
3. Alam M, Sultana M, Nair GB, Siddique AK, Hasan NA, Sack RB, Sack DA, Ahmed KU, Sadique A, Watanabe H, Grim CJ (2007) Viable but nonculturable *Vibrio cholerae* O1 in biofilms in the aquatic environment and their role in cholera transmission. Proc Natl Acad Sci USA 104: 17801-17806.
4. Farugue SM, Albert MJ, Mekalanos JJ (1998) Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. Microbiol. Mol. Biol. Rev 62: 1301-1314.

5. Safa A, Nair GB, Kong RY (2010) Evolution of new variants of *Vibrio cholerae* O1. Trends Microbiol 18: 46–54.
6. Nair GB, Qadri F, Holmgren J, Svennerholm AM, Safa A, Bhuiyan NA, Ahmad QS, Faruque SM, Faruque AS, Takeda Y, Sack DA (2006) Cholera due to altered El Tor strains of *Vibrio cholerae* O1 in Bangladesh. J Clin Microbiol 44: 4211–4213.
7. Chin CS, Sorenson J, Harris JB, Robins WP, Charles RC, Jean-Charles RR, Bullard J, Webster DR, Kasarskis A, Peluso P, Paxinos EE (2011) The origin of the Haitian cholera outbreak strain. N Engl J Med 364: 33–42.
8. Weill FX, Domman D, Njamkepo E, Almesbahi AA, Naji M, Nasher SS, Rakesh A, Assiri AM, Sharma NC, Kariuki S, Pourshafie MR (2019) Genomic insights into the 2016–2017 cholera epidemic in Yemen. Nature 565: 230-233.
9. Naha A, Pazhani GP, Ganguly M, Ghosh S, Ramamurthy T, Nandy RK, Nair GB, Takeda Y, Mukhopadhyay AK (2012) Development and evaluation of a PCR assay for tracking the emergence and dissemination of Haitian variant *ctxB* in *Vibrio cholerae* O1 strains isolated from Kolkata, India. J Clin Microbiol 50: 1733–1736
10. Taneja N, Mishra A, Sangar G, Singh G, Sharma M (2009) Outbreaks caused by new variant of *V. cholerae* O1 El Tor, India. Emerging Infect Dis 15: 352–353.
11. Pal BB, Khuntia HK, Nayak SR, Mohanty A, Biswal B (2017) *Vibrio cholerae* O1 Ogawa strains carrying the *ctxB7* allele caused a large cholera outbreak during 2014 in the tribal areas of Odisha, India. Jpn J Infect Dis 70: 549–553.
12. Pal BB, Khuntia HK, Samal SK, Kerketta AS, Kar SK, Karmakar M, Pattnaik B (2013) Large outbreak of cholera caused by El Tor variant *Vibrio cholerae* O1 in the eastern coast of Odisha, India during 2009. Epidemiol Infect 141: 2560–2567.
13. Pal BB, Mohanty A, Biswal B, Nayak SR, Das BK, Lenka PP (2021) Haitian variant *Vibrio cholerae* O1 Ogawa caused cholera outbreak in Odisha. Indian J Med Microbiol 39: 513-517.
14. Nayak SR, Nayak AK, Biswal BL, Pati S, Pal BB (2020) Spread of Haitian variant *Vibrio cholerae* O1 causing cholera outbreaks in Odisha, India. Jpn J Infect Dis 74: 137-143.
15. Baur AW, Kirby WM, Sherris JC, Turch M (1966) Antibiotic susceptibility testing by a standardized single disc method. Am J Clin Pathol 45: 493–496.
16. Ghosh P, Naha A, Pazhani GP, Ramamurthy T, Mukhopadhyay AK (2014) Genetic traits of *Vibrio cholerae* O1 Haitian isolates that are absent in contemporary strains from Kolkata, India. PloS One 9:1129-1173.
17. Kumar P, Jain M, Goel AK, Bhadauria S, Sharma SK, Kamboj DV, Singh L, Ramamurthy T, Nair GB (2009) A large cholera outbreak due to a new cholera toxin variant of the *Vibrio cholerae* O1 El Tor biotype in Orissa, Eastern India. J Med Microbiol 58: 234–238.
18. Ramachandran D, Bhanumathi R, Singh DV (2007) Multiplex PCR for detection of antibiotic resistance genes and the *SXT* element: application in the characterization of *Vibrio cholerae*. J Med Microbiol 56: 346–351.
19. Cooper KL, Luey CK, Bird M, Terajima J, Nair GB, Kam KM, Arakawa E, Safa A, Cheung DT, Law CP, Watanabe H (2006) Development and validation of a pulse net standardized pulsed-field gel electrophoresis protocol for subtyping of *Vibrio cholerae*. Food Pathog Dis 3: 51–58.
20. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 33: 22-33.
21. Brick T, Primrose B, Chandrasekhar R, Roy S, Muliyil J, Kang G (2004) Water contamination in urban south India: household storage practices and their implications for water safety and enteric infections. Int J Hyg Environ Health 207: 473-480.
22. Deepa EK, Prasad SR, Muninarayana C (2010) Cholera outbreak in a village of Kolar District, Karnataka: lost opportunities for prevention? J Acad Clin Microbiol 12: 19-28.
23. Sarkar R, Prabhakar AT, Manickam S, Selvapandian D, Raghava MV, Kang G, Balraj V (2007) Epidemiological investigation of an outbreak of acute diarrheal disease using geographic information systems. Trans R Soc Trop Med Hyg 101: 587—593.
24. Pal BB, Khuntia HK, Samal SK, Kar SK, Patnaik B (2010) Epidemics of severe cholera caused by El Tor *Vibrio cholerae* O1 Ogawa possessing the *ctxB* gene of the classical biotype in Orissa, India. Int J Infect Dis 14: 384–389.
25. Faruque AS, Alam K, Malek MA, Khan MG, Ahmed S, Saha D, Khan WA, Nair GB, Salam MA, Luby SP, Sack DA (2007) Emergence of multidrug-resistant strains of *Vibrio cholerae* O1 in Bangladesh and reversal of their susceptibility to tetracycline after two years. J Health Popul Nutr 25: 241–243.
26. Garg P, Sinha S, Chakraborty R, Bhattacharya SK, Nair GB, Ramamurthy T, Takeda Y (2006) Emergence of fluoroquinolone-resistant strains of *Vibrio cholerae* O1 biotype El Tor among hospitalized patients with cholera in Calcutta, India. Antimicrob Agents Chemother 45: 1605–1606.
27. Goel AK, Jain M, Kumar P, Jiang SC (2010) Molecular characterization of *Vibrio cholerae* outbreak strains with altered El Tor biotype from southern India. World J Microbiol Biotechnol 26: 281–287.
28. Waldor MK, Tschäpe H, Mekalanos JJ (1996) A new type of conjugative transposon encodes resistance to sulfamethoxazole, trimethoprim, and streptomycin in *Vibrio cholerae* O139. J Bacteriol 178: 4157–4165.
29. Coote JG (1992) Structural and functional relationships among the RTX toxin determinants of Gram-negative bacteria. FEMS Microbiol Rev 8: 137–161.
30. Lin W, Fullner KJ, Clayton R, Sexton JA, Rogers MB, Calia KE, Calderwood SB, Fraser C, Mekalanos JJ (1999) Identification of a *Vibrio cholerae* RTX toxin gene cluster that is tightly linked to the cholera toxin prophage. Proc Natl Acad Sci USA 96: 1071–1076.

Corresponding author

Bibhuti Bhusan Pal, MSc, PhD
Scientist-G, Head of Microbiology Division, ICMR-Regional Medical Research Centre,
Chandrasekharpur, Bhubaneswar – 751023, India
Tel: +91-674-2305638
Fax: +91-674-2301351
Email: bbpal_rmrc@yahoo.co.in

Conflict of interests: No conflict of interests is declared.