Large outbreak of cholera caused by El Tor variant *Vibrio cholerae* O1 in the eastern coast of Odisha, India during 2009

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SUMMARY

A large outbreak of cholera reported during April–July 2009 in the Kendrapada district of Odisha, India was investigated. Forty-one rectal swabs and 41 water samples, collected from diarrhoeal patients and from different villages were bacteriologically analysed for the isolation of bacterial enteriopathogens, antibiogram profile and detection of various toxic genes. The bacteriological analysis of rectal swabs and environmental water samples revealed the presence of *V. cholerae* O1 Ogawa biotype El Tor. The *V. cholerae* strains were resistant to ciprofloxacin, co-trimoxazole, chloramphenicol, streptomycin, ampicillin, furazolidone and nalidixic acid. The multiplex polymerase chain reaction (PCR) assay on *V. cholerae* strains revealed the presence of *ctx*A and *tcp*A genes. The mismatch amplification of mutation assay (MAMA) PCR on clinical and environmental isolates of *V. cholerae* revealed that the strains were El Tor biotype, which harboured the *ctx*B gene of the classical strain. The random amplified polymorphic DNA PCR analysis and pulsed-field gel electrophoresis results indicated that the *V. cholerae* isolates belonged to the same clone. This investigation gives a warning that the El Tor variant of *V. cholerae* has spread to the coastal district causing a large outbreak that requires close monitoring and surveillance on diarrhoeal outbreaks in Odisha.

Key words: El Tor variant *Vibrio cholerae*, Odisha, spread.

INTRODUCTION

The disease cholera, caused by *Vibrio cholerae*, has been reported throughout the world and is of primary importance in developing countries. This disease is endemic, epidemic and pandemic in nature in modern times. *V. cholerae* has many serogroups and serotypes.

Of the serogroups mainly *V. cholerae* O1 and O139 are the outbreak/epidemic strains. *V. cholerae* O1 has two biotypes (classical and El Tor), which are traditionally distinguished by phenotypic tests and genotypic differences. However, three variants of the El Tor biotype have been described recently: Matlab (in Bangladesh) variants in 2002 which could not be biotyped because they possessed a mixture of the characteristics of both classical and El Tor [1]; Mozambique variant in 2004–2005, which has a typical El Tor genome but a tandem repeat of the classical ctx prophage in the small chromosome [2]; and El Tor variant

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(a typical El Tor biotype and an El Tor ctx prophage that produces ctx of the classical type); predominant in Bangladesh since 2001 [3]. The El Tor variant of *V. cholerae* has also been described in other regions of Asia and Africa [4]. The *V. cholerae* strain, responsible for the expanding cholera epidemic in Haiti during 2010 was due to El Tor variant O1 strains that are also predominant in South Asia, including Bangladesh [5, 6]. Similarly, outbreaks of cholera caused by a strain of the *V. cholerae* O1 El Tor variant have been reported in Punjab and Haryana during July–September 2007 [7]. Large outbreaks of cholera have also been reported from South India [8]. Epidemics of severe cholera caused by El Tor variants of *V. cholerae* O1 Ogawa in Odisha were reported from six tribal blocks of three districts of Odisha during July–September 2007 [9]. In this study we investigated the causative agent of the diarrhoeal outbreak, the antibiogram profile and detection of various toxic genes of *V. cholerae* and their clonality, which were reported during April–July 2009 in the Rajnagar block of Kendrapada district, on the eastern coast of Odisha, India.

METHODS

Case definition

A case of severe diarrhoea was defined as the occurrence of $\geqslant 3$ watery stools in a 24-h period associated with a clinical history of vomiting, abdominal cramping and severe dehydration in residents of Rajnager block between April and July 2009.

Data collection

An investigating team from the Microbiology Department, Regional Medical Research Centre (RMRC), Bhubaneswar and local health workers from the Rajnagar Community Health Centre (CHC) searched actively to identify severe diarrhoeal cases in the diarrhoea-affected villages. They collected a clinical history of diarrhoeal patients, and risk factors for the acquisition and spread of the infection in that block. All severe diarrhoeal cases were recorded and plotted in an epidemic curve to check the progress of the outbreak. Simultaneously, severe diarrhoeal cases were checked by month for the last 3 years from the hospital records to confirm the diarrhoeal outbreak. The occurrence of severe diarrhoeal cases was plotted in the block map by month to observe

the spread of the disease. Prior to onset of the diarrhoeal outbreak in Rajnagar block a large festival had been held on 14 April 2009, with about 30 000 people attending between 08:00 and 18:00 hours with some returning later in the evening. Suspecting the festival as the source of infection detailed information was collected regarding the festival; e.g. types of food, source of drinking water available at the festival. The first reported diarrhoeal patient was identified from the hospital record and traced back to her village. This person had returned to her village from the local festival and after 24 h developed severe diarrhoea associated with vomiting, abdominal cramping and severe dehydration. A house-to-house survey was conducted in the villages using a questionnaire to collect information on the villagers' knowledge, attitude and practices regarding diarrhoea as well as socio-demographic information on the community in the diarrhoea-affected villages.

Sample collection

Rectal swabs from severe diarrhoeal patients were collected in Cary–Blair transport medium from Rajnagar CHC and also from the households of diarrhoea-affected villages of Rajnagar block, Kendrapada district. The rectal swabs were collected from patients prior to the administration of antibiotics. The samples were transported to the RMRC, Bhubaneswar within 24 h of collection for bacteriological analysis. After isolation of *V. cholerae* from the rectal swabs as the aetiological agent of the outbreak, water samples were collected non-randomly from water bodies, e.g. ponds, rivers, small roadside water reservoirs as well household water from various different diarrhoea-affected villages in order to determine the source of infection.

Processing of samples

Rectal swabs were inoculated onto MacConkey agar, Hektoen enteric agar (HEA), thiosulphate-citrate-bile-salt sucrose agar (TCBS) and enrichment was performed in Selenite-F broth and alkaline peptone water (APW) [10]. Water samples were processed through membrane filtration, sedimentation and enriched in double-strength APW medium and then subcultured on TCBS plates to check for growth of *V. cholerae* strains [9]. *V. cholerae* was identified by standard biochemical tests and serotyping was performed using antisera obtained from Becton Dickinson (BD, USA).

Control measures

All the drinking water sources from different villages in the Rajnagar area were chlorinated. A public awareness campaign was initiated based at sub-centres located in various villages. The sub-centre staff were instructed to refer all cases of diarrhoea to Rajnagar CHC. Intravenous fluids, oral rehydration solution and essential drugs were stocked at the sub-centres. Pharmacists and nurses at the sub-centres were instructed to provide intravenous fluid while referring cases to the PHC.

Antimicrobial susceptibility testing

The sensitivity and the resistance pattern of *V. cholerae* O1 strains were tested with antibiotic-impregnated commercial disks (Himedia, India) using ampicillin (10 μg), chloramphenicol (30 μg), co-trimoxazole (25 μg), ciprofloxacin (5 μg), furazolidone (100 μg), gentamicin (10 μg), neomycin (30 μg), nalidixic acid (30 μg), norfloxacin (10 μg), streptomycin (10 μg), tetracycline (30 μg), azithromycin (15 μg) and poly-myxin B (50 μg). *V. cholerae* O1 strains were sub-cultured in tryptic soy broth (BD, USA) and plated on Mueller–Hinton agar (BD, USA). Plates were incubated for 24 h at 37 °C. Characterization of strains as susceptible or resistant was determined based on the size of the inhibition zones around each antibiotic disk according to the manufacturer's instructions following the Kirby–Bauer technique [11].

Polymerase chain reaction (PCR) assay

A multiplex PCR-based assay was employed to determine the presence of the A subunit of the cholera toxin gene (*ctx*A) and to biotype the *V. cholerae* strains by targeting *tcp*A (encoding the major structural subunit of the toxin co-regulated pilus), which is specific for El Tor and classical strains, according to the method described by Khuntia *et al.* [12].

Mismatch amplification mutation assay (MAMA)-PCR for differentiation of the cholera toxin B subunit of classical and El Tor biotypes of *V. cholerae* O1

The MAMA-PCR was designed to detect the nucleotide sequence difference at position 203 of the *ctx*B gene for the identification of cholera toxin of classical and El Tor biotypes. For this, a conserved forward primer (FW-com) and two allele-specific primers, Rv-cla and Rv-elt, were designed that are able to

Table 1. *Severe diarrhoea in Rajnagar block, Kendrapada district, April–July 2009*

	Number
Total gram panchayats	18
Diarrhoea-affected gram panchayats	16
Total villages	224
Diarrhoea-affected villages	108
Total population	162 984
Diarrhoea-affected population	96 878

amplify *ctx*B of classical and El Tor biotypes, respectively [13]. Using the above technique the environmental and clinical isolates of all *V. cholerae* O1 strains were subjected to simplex PCR assay to detect the *V. cholerae* O1 El Tor variant that harbours the classical *ctx*B gene and *V. cholerae* O1 that harbours the El Tor *ctx*B gene separately. The random amplified polymorphic DNA (RAPD) PCR assay was performed on selected *V. cholerae* strains with 1281 and 1283 primers to check the clonality as in a previous study [14].

Pulsed-field gel electrophoresis (PFGE) analysis

Genomic DNA of *V. cholerae* O1 was prepared in agarose plugs to perform PFGE. As described by Sinha *et al.* [15], PFGE was performed using the counter-clamped homogenous electric field method on the CHEF Mapper system (Bio-Rad, USA) with 1% PFGE grade agarose in 0·5× TBE buffer. Run condition was generated by an auto-algorithm mode of CHEF Mapper, the PFGE system was comparable to a size range of 20–300 kb markers for *V. cholerae* strains. The gel was stained in 10 μg/ml ethidium bromide solution for 30 min, de-stained in water for 15 min and photographed under UV light in an Alpha Imager (Alpha InfoTech Corporation, USA).

RESULTS

The diarrhoeal cases were reported to Rajnagar CHC on 15 April 2009 at midnight. The diarrhoeal patients that had attended the festival originated from different villages. According to data available from Rajnagar CHC, of the total 18 gram panchayats (GP; rural local governments), 224 villages and 162 984 inhabitants there were 16 GP, 108 villages and 96 878 inhabitants that were affected by severe cases of diarrhoea, respectively (Table 1). Of these, 715 severe diarrhoeal cases and seven deaths were reported with an attack
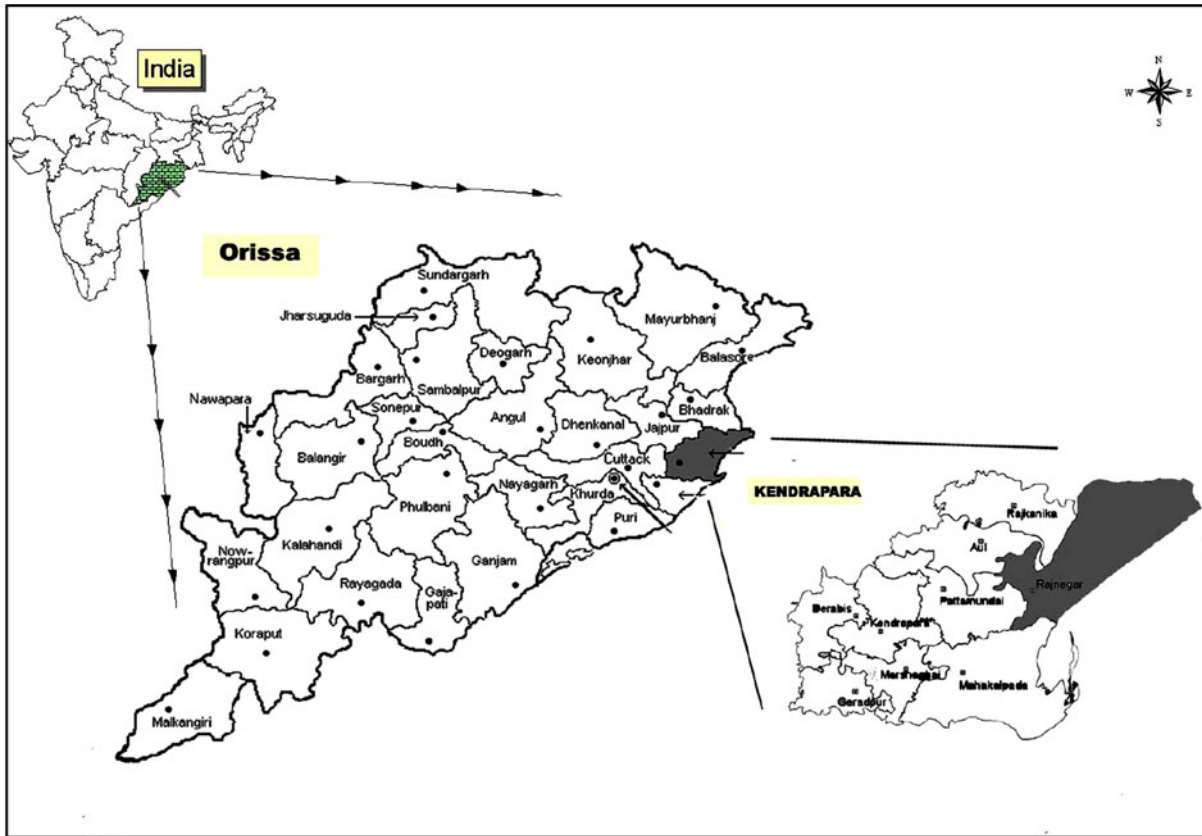
Fig. 1 [*colour online*]. Diarrhoea-affected Rajnagar block, Kendrapada district (April–July 2009).

rate of 0·7% and a case-fatality rate of 0·98%. Diarrhoea-affected villages and cases were analysed by month. This analysis indicated that the highest number of diarrhoea-affected villages were reported during April ($n = 63$) followed by May ($n = 41$), June ($n = 3$) and July ($n = 1$) (Figs 1, 2). Figure 3 depicts all cases of diarrhoea according to date. Interestingly, the epidemic curve produced many peaks, which indicates that the clustering of cases was reported in different time periods from different villages. After appropriate control measures initiated by the government of Odisha, the number of cases of diarrhoea declined gradually and after 8 July 2009 no further cases were reported.

The rectal swabs collected from diarrhoeal patients from Rajnagar CHC and also from the diarrhoea-affected villages were analysed bacteriologically. The water samples from the ponds, tube wells and roadside water reservoirs from 12 affected villages were analysed for the presence of *V. cholerae*. Of 41 rectal swabs, 27 were culture positive and of these, 18 (67%) were *V. cholerae* O1 Ogawa biotype El Tor, followed by seven (26%) *E. coli* and two

(7%) *Aeromonas* spp. (Table 2). Similarly, 2/41 water samples collected from different ponds, roadside small water reservoirs, rivers, and tube well water were analysed and the pond water tested positive for *V. cholerae* O1 Ogawa (Table 3). The antibiogram profile of *V. cholerae* isolates isolated from diarrhoeal patients and the pond water indicated that the *V. cholerae* strains were sensitive to tetracycline, norfloxacin, gentamicin, azithromycin, neomycin, erythromycin and resistant to ciprofloxacin, co-trimoxazole, chloramphenicol, streptomycin, ampicillin, nalidixic acid and furazolidone. The molecular analysis of all *V. cholerae* strains isolated from diarrhoeal patients and pond water samples revealed that the El Tor variants of *V. cholerae* O1 Ogawa with the *ctxB* gene of the classical strain was the causative organism of this outbreak, which was confirmed by MAMA-PCR. The RAPD-PCR analysis by 1281 and 1283 primers on representative *V. cholerae* isolates indicated that they belonged to same clone. Similarly, the PFGE analysis on a number of representative strains along with the 2007 tribal cholera epidemic strain revealed them to be clonal in nature (Fig. 4).
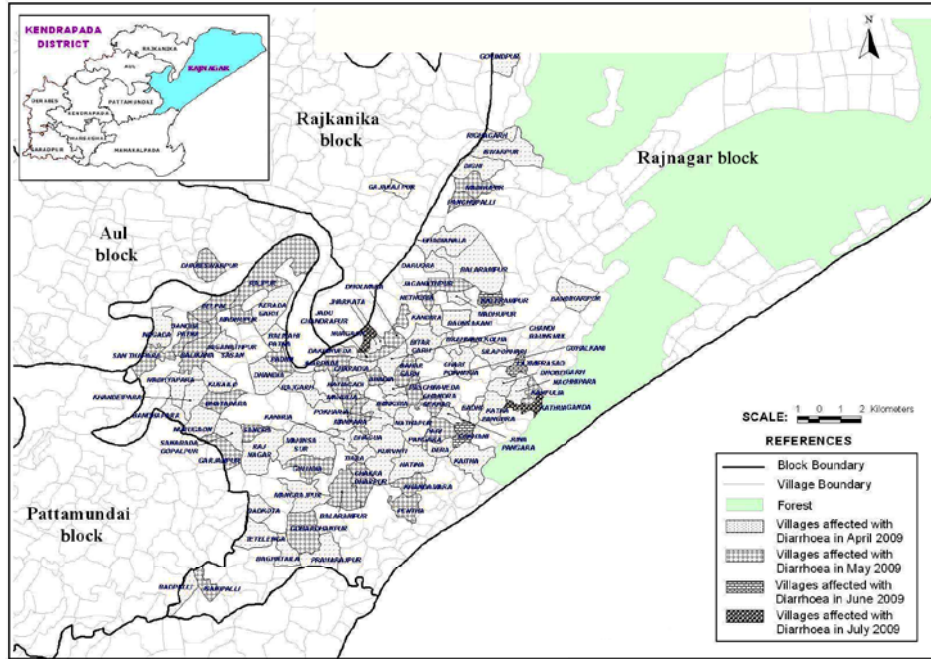
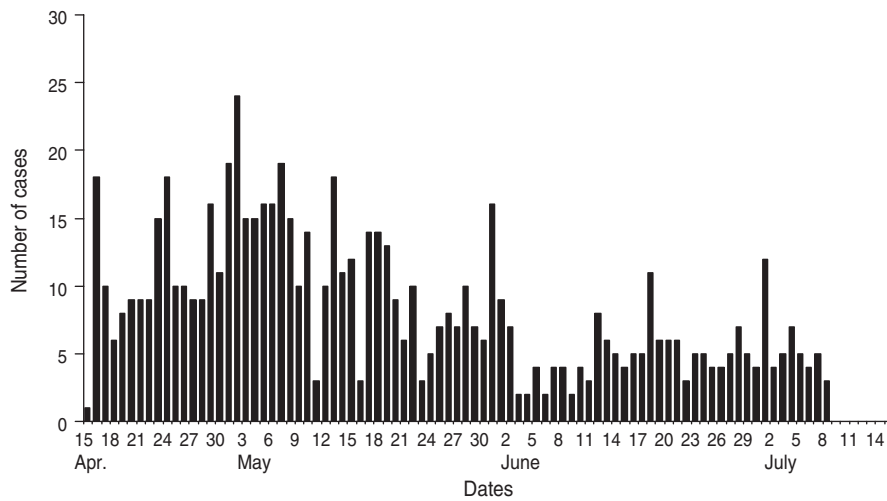**Fig. 2** [*colour online*]. Diarrhoea-affected villages in Rajnagar block by month (April–July 2009).

Fig. 3. Number of cases of severe diarrhoea by date, Rajnagar block, April–July 2009.

Table 2. *Bacteriological analysis of enteropathogens isolated from diarrhoeal patients*

	No. (%)
Total samples	41 (100)
Culture negative	14 (34)
E. coli	7 (26)
V. cholerae O1 Ogawa	18 (67)
Salmonella spp.	0
Shigella spp.	0
Aeromonas spp.	2 (7)

Table 3. *Analysis of water samples collected from Rajnagar block*

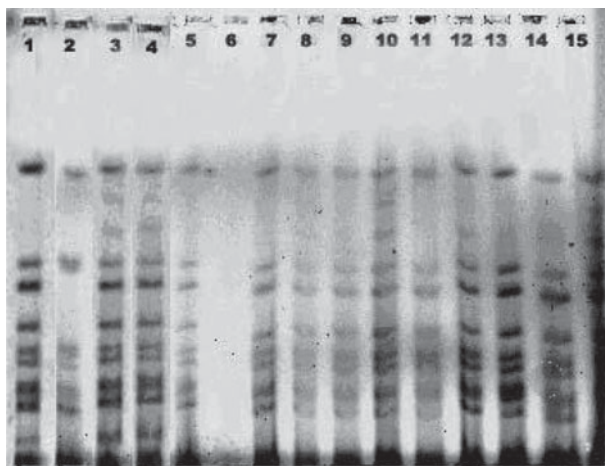| Sample no.	Category	Total samples	No. positive for *V. cholerae* O1 Ogawa (%)
1	Pond	29	2 (7)
2	Road side small reservoir	4	0
3	River	1	0
4	Tube well	7	0
	Total	41	2 (5)

Fig. 4. PFGE analysis of *Vibrio cholerae* O1 Ogawa isolates during 2000, 2007 and 2009. 1, 569B-*V.cholerae* O1 classical; 2, VC 20-*V. cholerae* O1 El Tor; 3, 1669N-El Tor variant *V. cholerae* with *ctx*B gene of classical strain; 4, DM8 (2007); 5, JP47 (2000); 6, water; 7, RJ15 (2009); 8, RJ7 (2009); 9, KP3 (2007); 10, RJ9 (2009); 11, RJ10 (2009); 12, RJ16 (2009); 13, DM7 (2007); 14, KP14 (2007), 15, DM1 (2007).

DISCUSSION

Cholera, a waterborne disease, is transmitted due to contamination of water sources. The present cholera outbreak might have originated from the festival during April 2009 and continued for 3 months up to July 2009 affecting many villages. The water samples collected from the pond water were positive for *V. cholerae* O1 Ogawa and therefore contact with pond water is a possible source of infection. Analysis of samples of severe diarrhoeal patients from Rajnagar CHC according to month and year (2006–2009) indicated that this was a definite outbreak of diarrhoea which occurred during April–July 2009. The early reporting of the causative organism, chlorination of different water sources and intervention of appropriate control measures stopped the spread of the disease to other adjacent blocks.

Cholera is one of the major health problems in Odisha leading to great morbidity and mortality. Since the last two decades several studies on diarrhoeal disorders have revealed the causative agents of cholera as *V. cholerae* O1 Ogawa/Inaba biotype El Tor, *V. cholerae* O139 and *V. choleare* O1 El Tor variant [9, 14]. During the super-cyclone in 1999 there was a cholera outbreak in Odisha in nine districts including Kendrapada district due to *V. cholerae* O1 Ogawa El Tor biotype and *V. cholerae* O139 serogroup [14]. The present large cholera outbreak in the Rajnagar area was due to an El Tor variant of *V. cholerae* O1 strains that carried the *ctx*B gene of the classical strain, which was detected for the first time in that area.

Since 1995 interesting fluctuations in the pattern of resistance to various antibiotics have been witnessed in *V. cholerae* O1 and O139 serogroups in Odisha. The antibiotic resistance profile of *V. cholerae* O1 and O139, isolated during the 1999 super-cyclone cholera outbreak was: ampicilin, co-trimoxazole, furazolidone, neomycin, nalidixicacid, streptomycin (for O1), and ampicilin, furazolidone, neomycin, streptomycin (for O139) [14]. One hundred percent nalidixic acid-resistant *V. cholerae* O139 and 100% ampicilin-susceptible *V. cholerae* O1 have been reported from 2000 onwards. Although emergence of fluoroquinolone-resistant *V. cholerae* were reported before 2007, 64·8% ciprofloxacin- and 63·2% norfloxacin-resistant *V. cholerae* were isolated during the 2007 cholera outbreak where tetracycline was the best alternative drug of choice [9]. In the present study, with the exception of norfloxacin, a high percentage of resistance to ciprofloxacin, ampicillin, nalidixic acid, furazolidone, streptomycin and chloramphenicol has been observed which is in accord with reports during the 2007 cholera epidemic in the tribal areas of Odisha [9]. Similar cholera outbreaks have been reported during 2004 from Southern India [16]. Multiple antibiotic resistance in *V. cholerae* has emerged as a major problem worldwide [17]. Since 1996 there has been a progressive increasing trend of antibiotic resistance towards common fluroquinolones in India, i.e. ciprofloxacin and norfloxacin [9, 18–20]. The *V. cholerae* strains isolated from this outbreak were sensitive to tetracycline which is different from reports in other regions of India [21]. Multidrug resistance prevents additional changes to disease management. Some antibiotics are deemed unusable for certain population groups, i.e. tetracycline is not recommended for use in children and quinolone is not recommended for pregnant women and children [22]. These rapid shifts in antimicrobial resistance are in contrast to the previous decade when antibiotic resistance in *V. cholerae* O1 was not a usual phenomenon and multiple antiobiotic-resistant strains were rare. However, this pattern of quick shifts in antibiogram profile indicates an enhanced mobility in genetic elements, which confers resistance to antibiotics. Recent studies by Beaber & Waldor [23] have shown that SXT constin, a mobile genetic element that is a conjugative self-transmissible plasmid, is responsible

for antibiotic resistance. Recent studies suggest SXT constin as an important element for horizontal dissemination of antibiotic-resistant genes in bacteria. In SXT constin, the antibiotic resistance genes are clustered in certain frameworks. These genes confer resistance to chloramphenicol, sulfamethaxazole, streptomycin and trimethoprim [24]. Therefore, it can be assumed that *V. cholerae* strains might harbour SXT constin as reported by Goel *et al.* [16].

Cholera pathogenesis is a complex process, which involves the synergetic action of several genes. Cholera toxin, constituting AB subunits is considered as the most important epidemic marker in various toxins produced by *V. cholerae* [25]. Several studies have indicated that there is continual change in the sequence of the *ctx*B gene. Biotype-specific ctxφ is found in *V. cholerae* strains. El Tor biotype strains harbour ctx^{ElT}φ while classical strains have ctx^{class}φ. However, the genomic structure of ctxφ, in which the cholera toxin genes are maintained, differs between the classical and El Tor biotypes [2, 26]. Interestingly, the new *V. cholerae* O1 variants carrying the *ctx*B gene of the classical strains were first isolated in Bangladesh in patients with severe diarrhoea during 2002. In this study all the El Tor *V. cholerae* isolates possessed the *ctx*B gene of classical biotype and belonged to the same clone of *V. cholerae* strains that caused the 2007 cholera epidemic in Odisha (Fig. 4). Similar results with El Tor variant *V. cholerae* strains causing outbreaks and epidemics of cholera in different countries in Asia, USA, and Africa have been reported [4–6, 9, 18]. Studies from India, Bangladesh and USA show that the classical cholera toxin-producing El Tor strains are now replacing the seventh pandemic El Tor strains [5, 6, 8, 9]. The El Tor variant *V. cholerae* with classical *ctx*B trait, spread to the Rajnagar area and caused a large cholera outbreak subsequent to the 2007 cholera epidemic in the southern tribal districts in Odisha.

The present study indicates that after the large cholera epidemic of 2007 in the tribal areas of Odisha, the epidemic spread to the coastal areas with a high percentage of antibiotic resistance. The present cholera outbreak is an early warning that future outbreaks/epidemics of diarrhoeal disorders in Odisha may occur due to this El Tor variant of *V. cholerae* O1 strain. This requires implementation of continuous surveillance and monitoring of diarrhoeal outbreaks/epidemics in different regions of Odisha and India.

DECLARATION OF INTEREST

None.

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