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Altered El Tor *Vibrio cholerae* O1 Caused Outbreak of Cholera in the Southern Part of Odisha, India during 2011

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Outbreak of cholera reported during July to October, 2011 from the Mohana block of Gajapati district of Odisha was investigated. Sixty rectal swabs collected from severe diarrhoea patients and 64 water samples were bacteriologically analyzed for the isolation of bacterial pathogens, antibiogram profile and detection of various toxic genes. Bacteriological analysis of rectal swabs and water samples detected *V.cholerae* O1 Ogawa biotype El Tor. The *V. cholerae* strains were resistant to streptomycin, erythromycin, ampicillin, furazolidone, co-trimoxazole nalidixic acid and chloramphenicol. The multiplex PCR assay on *V. cholerae* strains indicated the presence of ctxA and tcpA genes showing biotype El Tor; whereas mismatch amplification of mutation assay (MAMA) PCR assay on clinical and water isolates of *V. cholerae* revealed that they were El Tor variant carrying ctxB gene of the classical strain. This clearly indicates the homology of clinical and environmental isolates of *V.cholerae* isolated during the outbreak period. Early reporting enabled the state government to implement control measures to check the spread of the disease. The present findings clearly gives an warning that the altered El Tor *V.cholerae* O1 strains with classical traits spread in the tribal areas causing cholera outbreak that may repeat in future which needs close monitoring.

Key words: Cholera outbreak, Altered El Tor *V. cholerae,* Tribal area, Odisha.

Vibrio cholerae causing severe diarrhoeal disorders has appeared in epidemic proportions in many developing countries and is endemic in Asia, Africa and South America. The toxigenic *V. cholerae* sero group O1 has frequently been reported from Indian subcontinent. Sero group O1 is classified into two biotypes, classical and El Tor. The seventh and most recent pandemic of cholera was caused by the El Tor biotype. Several atypical (or named as altered El Tor) *V.cholerae*, with classical ctxB strains have been identified, including the atypical El Tor in Matlab, Bangladesh, between 1991 and 1994[1].Since 1995, hybrid ctxΦ isolate carrying El Tor rstR and classical ctxB has completely replaced the El Tor biotype in Kolkata, India[2]. Sporadic outbreaks of cholera due to El Tor variant strains containing classical ctxB have been reported from different places of India including Odisha. Large outbreaks of cholera were reported from Kolkata during 2009[3], Tamilnadu during 2006-2009[4], Chandigarh between 2002-2008[5], from Yavatmal Maharashtra[6], and from Belgaon during 2010[7] by *V.cholerae* O1 El Tor variants with altered traits. In the course of time the new pathogenic *V. cholerae* variants have emerged and spread throughout many Asian and African countries over the past decade.

The cholera cases caused by El Tor variants ctxB classical biotype reported in Mozambique Bangladesh and several countries of Asia and Africa[8]. Again *V. cholerae* O1 biotype El Tor producing Haitian variant cholera toxin (HCT) were reported in India with high case fatality rate. This HCT secretary *V. cholerae* strains were

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associated with severe cholera epidemics in Odisha, Western Africa and Haiti[6]. El Tor variant ctxB classical *V. cholerae* strains were reported for the first time from the state during 1995 from the coastal district of Odisha (unpublished).

The epidemics of cholera were reported due to El Tor variants of *V. cholerae* during 2007 from the tribal areas[9], from the costal district during 2009[10] and during 2010 from Rayagada district of the state (Communicated). Large outbreak of cholera was reported during July to October, 2011 from the Mohana block of Gajapati district from the southern part of Odisha. The present study has been envisaged to document the causative agent of this out break and its antibiogram profile for implementation of control measures to check the spread of the disease.

MATERIALS AND METHODS

Sample collection

Rectal swabs from severe diarrhoea patients were collected prior to the administration of any antibiotics in Cary Blair transport medium from Mohana community health centre (CHC), Gajapati district and also from the cholera affected villages during July to October, 2011. The samples were transported to microbiology division of Regional Medical Research Centre, Bhubaneswar within 24hrs of collection for bacteriological analysis. Simultaneously environmental water samples were also collected from the stream, river, chua, open well, tube well, nala and household water supplies from different affected villages to find out the possible source and spread of infection.

Processing of samples

Rectal swabs were inoculated onto MacConkey agar, Thiosulfate citrate bile salt sucrose (TCBS) agar and Hektoen enteric agar (HEA) and enrichment was done in Selenite F broth and alkaline peptone water (APW) procured from Beckton and Dickinson (BD,USA) . Similarly, water samples were processed through membrane filtration, sedimentation and enriched in double-strength APW medium and further sub-cultured on TCBS agar plates to look for the growth of *V. cholerae* strains. *V. cholerae* was identified by standard biochemical test. Sero typing was carried out for confirmation of *V. cholerae* using antisera obtained from BD (USA)[9].

Antimicrobial susceptibility testing

The sensitivity and the resistance patterns of *V. cholerae* O1 strains were tested with antibiotic-impregnated commercial disks (Hi-Media, Mumbai, 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 polymyxin B (50 µg). *V. cholerae* O1strains 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 in accordance with the manufacturer's instructions following the Kirby–Bauer technique (1966)[11].

Polymerase chain reaction (PCR) assay

A multiplex PCR-based assay was employed to determine the presence of the A-subunit of 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 by the method described by Keasler and Hall (1993)[12].

Mismatch amplification mutation assay (MAMA)-PCR to differentiate 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 of *V. cholerae*. For this, a conserved forward primer (FW-Com) and two allele-specific primers, Rv-cla and Rv-elt were designed that can amplify *ctx*B of classical and El Tor biotypes respectively. Using the above technique the clinical isolates of all *V. cholerae* O1 strains were subjected to simplex PCR assay to detect the *V. cholerae* O1 El Tor variant that harbors classical *ctx*B and *V. cholerae* O1 that harbors El Tor *ctx*B separately[13].

RESULTS

The data obtained from the Mohana CHC, revealed that the first severe diarrhoea case was reported on 1.7.2011from Kusumpali village of

Mohana block. She was 18 yr female who was suffering from loose motion, vomiting, rice water stool, abdominal pain with severe dehydration. The patients were admitted to the Mohana CHC and cured. Gradually the cases increased and spread to other villages of Mohana block. The clinical signs and symptoms of the diarrhoea patients were sudden belching of abdominal pain with 5-6 hr with rice watery stool, vomiting and rapid progress of severe dehydration. A total of 236 diarrhoea patients and only one death were reported between 1.7.11 to 14.10.11. The worst diarrhoea affected villages were Mohana, Pindiki, Bada Khani, Dhana khanka, Liliguda, Budring Damiputa and Birikut etc. The incidence of severe diarrhoea cases reported from different villages during July to October, 2011 is described in Fig. 1(a,b) which reveals different peaks in different time periods. Majority of the cholera patients were over 25 year's age. A total of 60 rectal swabs were collected from the hospitalized diarrhoea patients of Mohana CHC and also from diarrhoea affected villages during this period. Out of total 60 samples collected 57 samples (95%) were culture positive. Out of the culture positive samples *E. coli* were 10 (17.5%), *V.cholerae* O1 Ogawa 45(78.9 %), *Shigella flexnerae* 1(1.8%), Aeromonas sp.1(1.8%) (Table-1). Seven out of 64 water samples collected from different water sources from different villages were positive for *V.cholerae* O1 Ogawa (Table- 2 & Fig:2).

Table 1. Bacteriological analysis of enteropathogens isolated from diarrhoea patients

Total samples	60
Culture positive	57 (95%)
E.coli	10(17.5%)
Vibrio cholerae O1 Ogawa	45(78.9%)
Shigella spp.	01(1.8%)
Salmonella spp.	0(0%)
Aeromonas spp	01(1.8%)
Culture negative	3(5%)

Fig. 1(a). Map showing cholera affected Mohana block of Gajapati District, Odisha

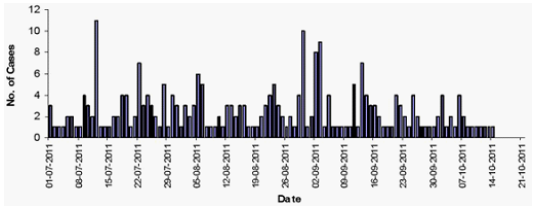

Fig. 1(b). Epi -curve showing date wise severe diarrhoea cases in Mohana block (1.7.11-21.10.11)

Table 2. Analysis of water samples collected from different villages for the presence of *V. cholerae*

S. No.	Category	Total samples	No. positive *V.cholerae* O1 Ogawa (%)	Isolation date and village name
1	Tube well	22	2 (9.1)	15.7.11 (Jhiliki) 18.8.11(Juba)
2	Open well	20	5(25%)	10.7.11 (Juba goan) 12.7.11 (Jhatika sahi) 13.7.11 (Chana bhania) 16.8.11(Badakhani) 21.8.11 (Dhanu pata)
3	Nala, chua	22	0(0%)	
Total		64	7(10.9%)	

Seven out of 64 water samples collected from river, stream, chua, nala, open well and dug well water were positive (tube well and open well) for *V. cholerae* O1 Ogawa biotype El Tor. First water sample was positive for *V. cholerae* O1 Ogawa collected from a tube well water of Jhiliki village on 15 .7.2011 where as the index case was detected on 1.7.2011 who was suffering from severe watery diarrhoea from Kusumpali village, hospitalized at Mohana CHC and the rectal swab collected from the index case was positive for *V. cholerae* O1 Ogawa. Then gradually more number of water samples were positive collected from open well and tube well water subsequently during the month of July and August, 2011(Table-2).Five number of water samples were positive for *V.cholerae* during July and 2 water samples during August, 2011 only. The *V.cholerae* O1 Ogawa strains were sensitive to gentamicin, ciprofloxacin, tetracycline, doxycycline, azithromycin; but were resistant to ampicilin, furazolidone, streptomycin, erythromycin, neomycin, chloramphenicol .The multiplex PCR assay showed that all *V.cholerae* O1 strains were positive for ctxA and tcpA genes showing biotype El Tor. However, the MAMA PCR assay for the detection of cholera toxin B submit of classical biotype of *V.cholerae* O1 indicated that all strains (isolated from stool and water)were El Tor variant that harbored the classical ctxB gene.

DISCUSSION

Contamination of water sources is the major cause of transmission of cholera. People usually go to paddy field during the rainy season, drink water from natural water sources like nala, chua, river and stream and get infection which is already published during 2007 cholera epidemic in Koraput ,Rayagada and Kalahandi districts[9] and during 2010 cholera epidemic from the Rayagada district also(unpublished). The same reason might be attributed for acquiring and spread of infection .This outbreak mostly confined to scheduled caste and scheduled tribe populations which are socially backward, mostly illiterate and poor. The people have disbelieves that while they suffer from diarrhoea they prefer to pray to local goddess and then consult the local quacks (disari-priest) for early treatment. But when the condition of the patient becomes worse to worst they seek the hospital treatment. This is one of the major causes of morbidity and mortality due to using unsafe drinking water and practicing poor hygienic practices found in the tribal areas[9]. The active surveillance and early reporting of pathogen which was isolated from stool samples and different water sources, to implement chlorination of different water sources and institution of appropriate control measures enabled by the state health authorities to check the spread of the disease in this block.

A high percentage of resistant to ampicilin, nalidixic acid, furazolidone, streptomycin, erythromycin, co-trimoxazole, neomycin and chloramphenicol of *V. cholerae* O1 Ogava strains were observed in the present study with contrast to the cholera epidemic of 2010 that occurred in the same block where *V. cholerae* O1 strains were resistant to tetracycline, chloramphenicol, norfloxacin, nalidixic acid, furazolidone and co-trimoxazole . The *V.cholerae* strains reported during 2010 cholera epidemic in the same area were 100% resistant to tetracycline (unpublished), which again

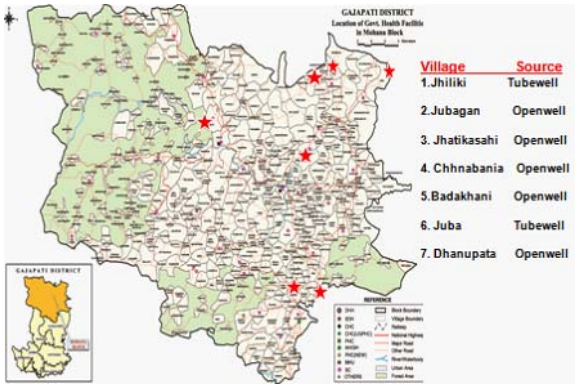

Fig. 2. Water sources positive for *V. cholerae* in Mohana block (July-Oct,2011)

become sensitive during 2011cholera outbreak reported in the present study. The lesser percentage of tetracycline resistant strains was isolated from this state during 2008-2009 (unpublished). The sudden change of tetracycline sensitivity during 2011 is unclear. Any increase in the antibiotics pressure in the community might be the reason for the emergence of such resistant strains. Extensive use of antibiotics without proper sensitivity testing and lack of surveillance program to monitor bacterial resistance in these areas might be the reason for emergence of multiple antibiotic resistances to *V.cholerae* strains.

The more number of water samples were found positive during the month of July 2011 which coincided with the occurrence of more number of diarrhoea cases during July – August 2011. This also corresponds with the incidence of severe diarrhoea cases where more cases were noticed during the month of July, 2011also. This is a clear cut indication that people were not using tube well and open well water hygienically which were contaminated and acted as career to spread the disease. Migration of people for attending the diarrhoea patients from nearby villages might be one of the reasons for spread of the disease. Similar results were also published from our earlier findings from the Rajnagar cholera epidemic during 2009 where pond water was contaminated and acting as a career to spread the disease[10]. Similar findings were also noticed during 2007 cholera epidemic in the tribal areas of Odisha[9].

The molecular analysis on the clinical and water isolates of *V.cholerae* O1 strains by multiplex PCR assay revealed that those isolates were positive for *ctx*A and *tcp*A genes showing biotype El Tor ; whereas the MAMA PCR assay revealed that they were El Tor variants of *V. cholerae* harboring *ctx*B gene of classical strain. The 2010 cholera outbreak in Mohana area was confined to few villages accounting for limited number of cases which was end part of the large cholera outbreak of Rayagada district during 2010 (Unpublished). But 2011 cholera outbreak in Mohana area again reappeared accounting for more morbidity which might be due to low immunity of people residing in that block.

It is very difficult to predict the origin of infection; but this might be due to infection from environmental water sources. Water sources like stream and open well water were positive and gradually spread to unaffected areas infecting more number of people. Large cholera epidemic was reported from Kasipur area of Rayagada district during 2007[10], again reappeared during 2010 in Rayagada district affecting Mohana block towards the end part of the cholera epidemic[16]. Then again reemerged and caused large cholera outbreak during 2011. The origin of the El Tor variant *V. cholerae* strain noticed in this state during 1995 from the coastal areas. Gradually those strains percolated in different parts of the state from 1995-2007; but caused the epidemic during 2007 by El Tor variant *V. cholerae* O1 strains. During the subsequent years the El Tor variant *V. cholerae* strains circulated in different districts of Odisha during 2008-2009[14]. The Haitian El Tor variant strain was reported from Odisha, South East Asia, and Eastern Kolkata where the clinical severity was noticed very high among patients[6]. The clinical severity of the patients was very worse observed in this study, which was also noticed during 2007 cholera epidemic in the tribal areas due to this El Tor variant *V. cholerae* O1 strains[10]. This is also supported by the report of Siddique, et al. (2010)[15]. Similar clones of *V. cholerae* might have circulated in the Mohana area or might be different which needs more analysis by PFGE and sequencing etc. Again the *V. cholerae* strains isolated over two decades from the state from different cholera outbreaks /epidemics should be analyzed thoroughly which will give more important information on molecular epidemiology that may help planning for the control of future outbreaks of cholera in this region.

Here we report the emergence of El Tor variants of *V. cholerae* carrying classical ctxB with multiple drug resistant strains causing large outbreak of cholera during 2011 in the southern part of Odisha dominated by tribal population. The present cholera outbreak clearly indicates source and spread of infection where clinical and environmental water isolates of *V. cholerae* were similar as evidenced by MAMA PCR assay. The active surveillance and early reporting of the pathogen isolated from diarrhoea patients and water samples enabled the state health authorities to implement adequate control measures to combat the cholera outbreak in Mohana area. The El Tor variant of *V. cholerae* O1 strains carrying ctxB

classical traits completely replaced the normal El Tor *V.cholerae* O1 strains that may further spread to the non-cholera affected areas in the state. Therefore, a systematic surveillance study is required to monitor both stool and water samples together to check the presence of *V. cholera* in the tribal areas of Odisha.

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