

## Incidence and molecular analysis of *Vibrio cholerae* associated with cholera outbreak subsequent to the super cyclone in Orissa, India

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### SUMMARY

An epidemiological study was carried out to find out the aetiological agent for diarrhoeal disorders in the cyclone and flood affected areas of Orissa, India. Rectal swabs collected from 107 hospitalized diarrhoea patients were bacteriologically analysed to isolate and identify the various enteropathogens. Detection of toxic genes among *E. coli* and *V. cholerae* was carried out by polymerase chain reaction (PCR) assay. Of the 107 rectal swabs analysed, 72·3% were positive for *V. cholerae* O1 Ogawa, 7·2% for *V. cholerae* O139, 1·2% for *E. coli* (EAggEC) and 1·2% for *Shigella flexneri* type 6. Using multiplex PCR assay it was found that all *V. cholerae* isolates were *ctxA* positive and El Tor biotype. Strains of *V. cholerae* O1 were observed to be resistant to nalidixic acid, furazolidone, streptomycin, co-trimoxazole and ampicillin. Except for nalidixic acid, the resistance pattern for O139 was identical to that of O1 strains. Representative strains of *V. cholerae* were further characterized by randomly amplified polymorphic DNA (RAPD) analysis and ribotyping. Both O1 and O139 *V. cholerae* strains exhibited the R3 pattern of ribotype and belonged to a similar pattern of RAPD compared with that of Calcutta strains. Early bacteriological and epidemiological investigations have revealed the dominance of *V. cholerae* O1 among the hospitalized patients in cyclone affected areas of Orissa. Drinking water scarcity and poor sanitation were thought to be responsible for these diarrhoeal outbreaks. Timely reporting and implementation of appropriate control measures could contain a vital epidemic in this area.

### INTRODUCTION

Recent years have witnessed a resurgence in the global incidence of cholera with 94 countries including India reporting a high incidence of cholera cases to WHO [1]. The 1990s have also witnessed the unprecedented emergence of new serogroup *Vibrio cholerae* O139 Bengal, associated with epidemic cholera and sub-

sequently the spread of new clones of both O1 and O139 serogroups [2–5]. All these events made the aetiological role played by *V. cholerae* very complicated and difficult to perceive when the sporadic and epidemic cholera takes place.

The state of Orissa, situated in the east coast of the Indian subcontinent, experienced an unprecedented super-cyclone during October 1999, which continued for more than 48 h with high wind velocity and torrential rain affecting six districts of coastal saline tract. Thirty kilometres of land area from the coast

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was submerged after three successive tidal waves struck the coast causing an enormous destruction to life and property. There were innumerable carcasses of livestock and the corpses were not cleared up as the aid-forces could not reach the submerged and cyclone affected areas. The natural disaster left a huge number of people homeless. An official estimate of more than 97000 attacks and 81 deaths due to diarrhoea were reported during the 1 month period from 1 November to 1 December 1999 in these six cyclone-affected districts of Orissa which is much higher than the morbidity figures of the whole state in the past 3 years (source: Health Department, Government of Orissa). However, the outbreak of diarrhoea was concomitant and sporadic in nature. Soon after the cyclone, an investigation was undertaken to establish the aetiopathologic agents responsible for the sporadic outbreaks of the diarrhoea in the cyclone-affected areas. In addition, molecular analysis of the *V. cholerae* strains was also performed to identify the clonality and source of this outbreak.

## MATERIALS AND METHODS

### Specimen collection

During the period between 8 November 1999 and 8 December 1999 (post-cyclone period), 107 rectal swabs were collected from freshly admitted cases having acute diarrhoeal symptoms from different hospitals/Primary Health Centres (PHCs) in the cyclone-affected areas before any treatment was instituted by the local Government health authorities. Rectal swabs were transported to the Regional Medical Research Centre (RMRC) laboratory at Bhubaneswar in Cary Blair transport medium and processed within 3–7 h of collection for *V. cholerae* and other enteropathogens, such as *Escherichia coli* and *Shigella* sp. using standard technique [6].

### Bacteriology and serology

Rectal swabs were inoculated to MacConkey, Hektoon Enteric (Difco, USA) and Thiosulphate-Citrate-Bile-Sucrose agar (Eiken, Tokyo, Japan) plates. After incubation at 37 °C for 24–48 h, plates were examined for the suspected organisms. Typical colonies were inoculated onto a multi-test medium [7] to facilitate rapid presumptive identification of *V. cholerae* and other enteropathogens to triple sugar iron (TSI) agar (Difco) for incubation at 37 °C. *V. cholerae* strains that showed typical alkaline slant

and acid but reactions were further subjected to oxidase test and serology using slide agglutination with polyvalent and monovalent antisera specific for Ogawa, Inaba and O139 antisera prepared and supplied by National Institute of Cholera and Enteric Diseases (NICED), Calcutta, India. The isolates of *V. cholerae* identified as O1 and O139 were further analysed at molecular level [8–9]. All the strains of *V. cholerae* were analysed by polymerase chain reaction (PCR) assay. Six strains of O1 Ogawa and 1 strain belonging to the serogroup O139 were selected for molecular typing based on the antibiograms. Two strains of O1 and one strain of O139 isolated from cholera cases during pre-cyclone period were also included for comparison. The other significant enteropathogens were tested with commercially available specific antisera (Difco). For each sample, at least three lactose fermenting colonies from the MacConkey agar plates were tested individually on TSI agar for presumptive identification of *E. coli*. Small green colour colonies grown on Hektoon Enteric Agar (HEA) plates were further tested for *Shigella* sp. using TSI agar and serology. All the *V. cholerae* strains were stored in nutrient agar (Difco) stabs at room temperature. *E. coli* strains were stored in Luria Broth cultures (Difco) with 15% glycerol at –70 °C.

### Antimicrobial susceptibility

The sensitivity and the resistance patterns for both O1 and O139 strains were tested with antibiotic impregnated commercial disks (Hi-Media, Mumbai, India) using ampicillin (10 µg), chloramphenicol (30 µg), cotrimoxazole (25 µg), ciprofloxacin (5 µg), furazolidone (100 µg), gentamicin (10 µg), neomycin (30 µg), nalidixic acid (30 µg), norfloxacin (10 µg), streptomycin (10 µg) and tetracycline (30 µg). Strains of O1 and O139 were cultured in tryptic soya broth (Difco) and plated on Muller Hinton agar (Difco). Plates were incubated for 24 h at 37 °C. Characterization of strains as susceptible or resistant was based on the size of the inhibition zones around each antibiotic disk in accordance with the manufacturers' instruction following the Kirby–Bauer technique [10].

### DNA template preparation and polymerase chain reaction (PCR) assay

A multiplex PCR-based assay was employed to determine the presence of A-subunit cholera toxin

Table 1. PCR primer sequences and conditions used for the detection of genes specific for diarrhoeagenic *E. coli* and *V. cholerae* isolates

Group/ organism	Target gene or encoding region	Primer sequences (5'-3')	Amplicon size (bp)	PCR conditions*	Reference
<b>Simplex PCR</b>					
ETEC	<i>elt</i>	GGCGACAGATTATACCGTGC CGGTCTCTATATCCCTGTT	450	94 °C 1·0 min 55 °C 1·5 min 72 °C 1·5 min	11
	<i>est</i>	ATTTTAA/CTTTCTGTATTA/GTCTT CACCCGGTACAA/GGCAGGATT	190	94 °C 1·0 min 55 °C 1·5 min 72 °C 1·5 min	11
EPEC	<i>eae</i>	AAACAGGTGAAACTGTTGCC CTCTGCAGATTAACCCTCTGC	454	94 °C 1·0 min 55 °C 1·5 min 72 °C 1·5 min	12
	<i>bfpA</i>	AATGGTGCTTGCCTTGCTGC GCCGCTTTATCCAACCTGGTA	324	94 °C 1·0 min 56 °C 1·5 min 72 °C 1·5 min	13
	EAF	CAGGGTAAAAGAAGATGATAA TATGGGGACCATGTATTATCA	397	94 °C 1·0 min 60 °C 1·5 min 72 °C 1·5 min	14
<b>Multiplex PCR</b>					
EHEC	<i>stx1</i>	CAACACTGGATGATCTCAG CCCCCTCAACTGCTAATA	350	94 °C 1·0 min 55 °C 1·0 min 72 °C 1·0 min	15
	<i>stx2</i>	ATCAGTCGTCACACTACTGGT CTGCTGTCACAGTGACAAA	110		
	EAgg	CTGGCGAAAGACTGTATCAT CAATGTATAGAAATCCGCTGTT	630	94 °C 1·0 min 53 °C 1·0 min 72 °C 1·0 min	16
EAggEC	<i>ast</i>	CACAGTATATCCGAAGGC CGAGTGACGGCTTTGTAG	94		17
<i>V. cholerae</i>	<i>ctxA</i>	CTCAGACGGGATTTGTTAGGCACG TCTATCTCTGTAGCCCCTATTACG	301	94 °C 1·0 min 60 °C 1·5 min 72 °C 1·5 min	
	<i>tcpA</i> (Classical)	CACGATAAGAAAACCGGTCAAGAG ACCAAATGCAACGCCGAATGGAG	617		8
	<i>tcpA</i> (El Tor)	GAAGAAGTTTGTAAGAAGAACAC GAAGGACCTTCTTTCACGTTG	471		

\* 30 cycles consisting of denaturation, annealing, extension.

gene (*ctxA*) and to biotype the *V. cholerae* strains by targeting *tcpA* (encoding the major structural subunit of the toxin-coregulated pilus), which is specific for El Tor and classical strains by a method described earlier [8]. *E. coli* strains were screened for the presence of a variety of virulence genes such as *elt* (encoding heat-labile toxin) and *est* (encoding heat-stable toxin) [11] for enterotoxigenic *E. coli* (ETEC); *eae* (gene for enterocyte attachment and effacement) [12], *bfpA* (gene for bundle forming pilli) [13], and enteropathogenic *E. coli* (EPEC) adherence factor [14] for EPEC; *stx*<sub>1</sub> (encoding Shiga toxin 1) and *st* 2 (encoding Shiga toxin 2) [15] for enterohaemorrhagic

*E. coli* (EHEC); EAggEC (plasmid of enteroaggregative *E. coli*) [16] and *ast* (encoding stable toxin produced by enteroaggregative *E. coli* [17] for enteroaggregative *E. coli* (EAggEC). Template DNA was prepared from the culture grown in LB for overnight by boiling in a water bath for 10 min and instantly cooling on ice. PCR amplification was done with appropriate volumes of 10 × amplification buffer (500 mM KCl, 100 mM Tris-HCl, 15 mM MgCl<sub>2</sub>, [pH 8·3]), 2·5 mM each deoxynucleoside triphosphate, 10 pmol of each primer, 1·25 unit of *rTaq* DNA polymerase (Takara Shuzo, Otsu, Japan), and 5 μl of template. The reaction volume was adjusted to 25 μl

Table 2. Isolation of *V. cholerae* from different districts of Orissa after cyclone

District	Total samples collected			Age group		<i>V. cholerae</i>		
	M	F	Total	Paediatric (no. of positive/total)	Adult	O1	O139	Total (%)
Jagatsinghpur	39	25	64	13/25	28/39	40	01	41 (64.06)
Cuttack	8	14	22	01/01	11/21	10	02	12 (54.55)
Kendrapada	04	05	09	01/03	05/06	06	00	06 (66.67)
Jajpur	02	00	02	00/00	00/02	00	00	00 (0.00)
Puri	05	03	08	01/01	06/07	04	03	07 (87.50)
Balasore	00	02	02	00/00	00/02	00	00	00 (0.00)
Total	58	49	107	16/30	50/77	60	06	66 (61.7)

Table 3. List of different bacterial strains isolated from diarrhoea patients in the cyclone affected areas

Total stool samples/rectal swabs analysed	107
Culture positive	83 (77.5%)*
<i>V. cholerae</i> O1	60 (72.3%)
<i>V. cholerae</i> O139	6 (7.2%)
<i>E. coli</i>	16 (19.3%)
<i>Shigella flexneri</i> , type 6	1 (1.2%)
Culture negative	24 (22.5%)

\* Percentages are in parentheses.

using sterile triple distilled water. Simplex and multiplex PCRs were performed in an automated thermocycler (Biometra, Germany) for 30 cycles using conditions described in Table 1.

### Ribotyping

Selection of *V. cholerae* strains for ribotyping and RAPD was based on criteria, i.e. expression of identical antibiotic resistance profile which consist of ACoFzNNS and AFzNS for the serogroups O1 and O139 respectively. Strains having these profiles were randomly selected from different cyclone affected areas. A pre-cyclone strain belongs to O1 serogroup, (DJ35), three Calcutta strains of O1 serogroup representing ribotypes 1–3 (VC 20, VC 3 and CO 840 respectively) and one O139 strain (SG 24) isolated in Calcutta during 1992 epidemic were also included for comparative study. Genomic DNA was digested with *Bgl*I for ribotyping. The chromosomal DNA fragments were separated by agarose gel electrophoresis (0.8% gel) and southern hybridized on nylon membrane (Amersham, UK). *Bgl*I digested chromosomal DNA was probed for 16S and 23S rRNA (18) using a non-radioactive detection kit (ECL, Amersham, UK).

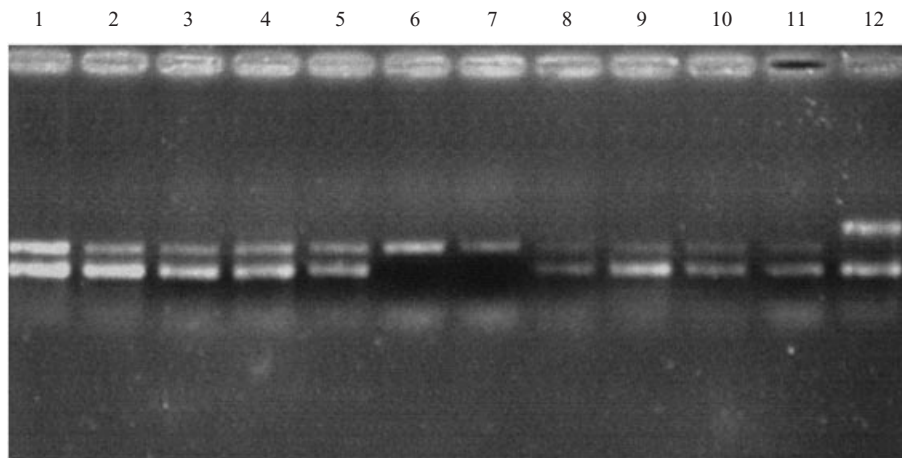
### Randomly amplified polymorphic DNA (RAPD) assay

RAPD was carried out in 25  $\mu$ l reaction mixture containing 2.5  $\mu$ l of 10 $\times$  PCR buffer (500 mM KCl, 100 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 150 mM MgCl<sub>2</sub>), 20 ng of genomic DNA, 2.5  $\mu$ l of 25 mM MgCl<sub>2</sub>, 20 pmol of primers 1281 (5'-AACG-CGCAAC) or 1283 (5'-GCGATCCCCA), 1 unit of Amplitaq DNA polymerase (Takara) and 2.5  $\mu$ l of 2.5 mM dNTP under a drop of mineral oil for 45 cycles of 94 °C for 1 min, 36 °C for 1 min, 72 °C for 2 min in an automated thermal cycler (Biometra) [19]. After PCR, 10  $\mu$ l of the products were electrophoresed in 1% agarose gel containing 5 mg/ml of ethidium bromide and photographed under UV light. The 1 kb DNA ladder (NEB, USA) was used as molecular size marker in all gels.

### RESULTS

The data collected from the Health Department, Government of Orissa revealed that, there were 97934 diarrhoea related attacks with 81 deaths during 1 month post-cyclonic period in the 6 affected districts as compared to 551 attacks with 43 deaths due to the same cause during the corresponding month of the preceeding year. However, there were clustering of cases of *V. cholerae* occurred in the worst affected Cuttack, Jagatsinghpur and Puri districts of Orissa (Table 2).

One hundred and seven rectal swabs collected during the post cyclonic period were bacteriologically and serologically analysed using standard methodology. The analysis revealed 83 culture positive cases with *V. cholerae* O1 Ogawa (72.3%), O139 (7.2%), *E. coli* (19.3%), *Shigella flexneri* (1.2%). Twenty-four samples (22.4%) remained culture negative for all the

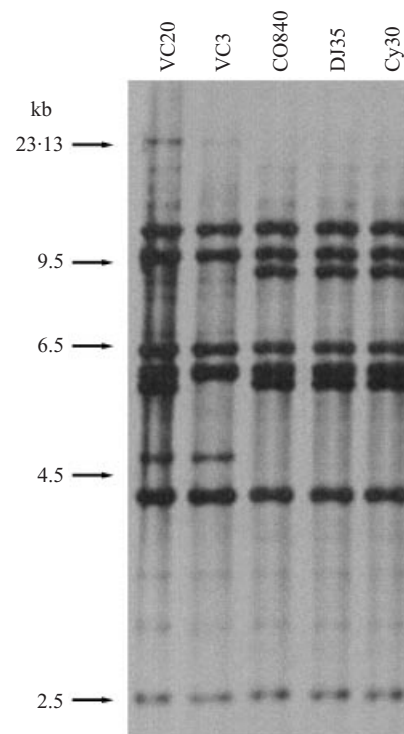


**Fig. 1.** PCR assay of the *ctxA* and *tcpA* genes of representative strains of *V. cholerae* O1 and O139 isolated before and after the cyclone. Lane 1, positive control for *ctxA* and *tcpA* El Tor, VC20; Lane 12, positive control for *ctxA* and *tcpA* classical, 569B; Lane 2–11, O1 and O139 strains isolated in Orissa before and after the cyclone. Lanes 6, 7, are pre-cyclonic strains which are *ctxA* negative and rest post-cyclonic strains which are *ctxA* positive.

tested enteric bacteria (Table 3). *V. cholerae* O1 were isolated more from males (53.03%) than from females (46.97%). The adults were more affected (75.76%) by *V. cholerae* than the paediatric age group (24.24%).

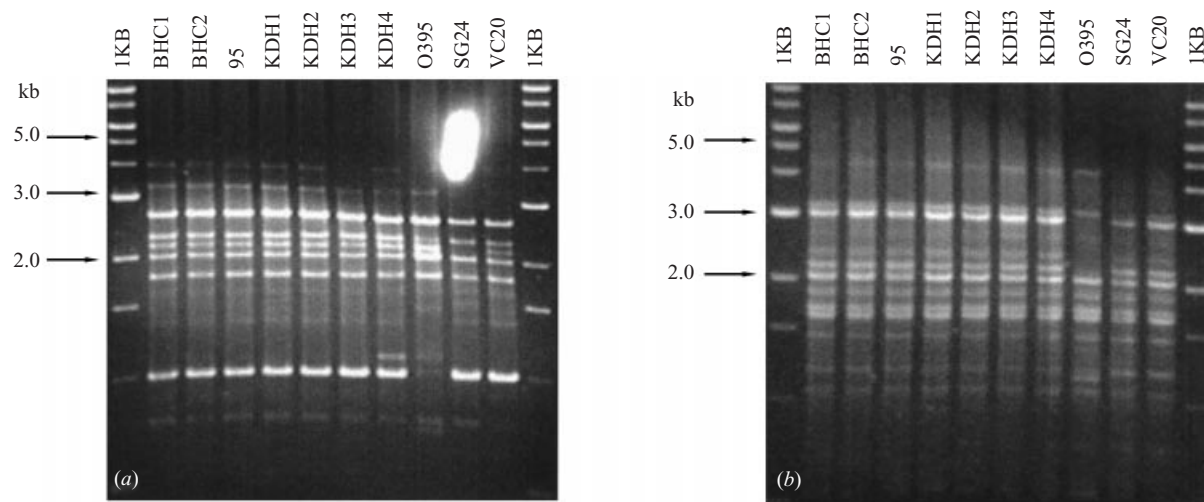
Fifty-nine *V. cholerae* O1 Ogawa and five O139 strains were analysed using the specific primers for the detection of A-subunit of the cholera toxin gene, *ctxA* and toxin coregulated pili gene *tcpA*, classical and El Tor by multiplex PCR. It was observed that all the tested *V. cholerae* O1 strains belonged to El Tor biotype and were positive for the *ctxA* and *tcpA* gene. All the *V. cholerae* O139 strains harboured the *ctxA* and *tcpA* specific for El Tor biotype (Fig. 1). The primers 1281 and 1283 were used for the RAPD analysis to detect their clonality, if any. Strains exhibiting identical antibiogram but representing different cyclone-affected areas were randomly selected for molecular epidemiological study using ribotyping and RAPD analysis. The antibiogram of *V. cholerae* O1 and O139 were ACoFzNNaS and AFzNS respectively. When tested with 16S and 23S rRNA probe, all the strains of *V. cholerae* exhibited ribotype R3 pattern (Fig. 2).

Like ribotyping results, all the *V. cholerae* O1 strains exhibited similarity with Calcutta strains especially with *V. cholerae* O1 that appeared after the O139 epidemic. Both the *V. cholerae* O139 strains exhibited similarity with SG 24; the strain isolated in Calcutta during O139 epidemic during 1992 (Fig. 3a, b). Two strains of *V. cholerae* did not agglutinate with O1 and O139 antisera. When tested by PCR for *ctxA* and O139 *rfb* genes, these were positive for both the genes. It would be worthwhile to characterize



**Fig. 2.** Ribotype pattern of representative O1 strains of *V. cholerae* isolated in Orissa before and after the cyclone. VC20, representative strain of R1 ribotype pattern; VC3, representative strain of R2 ribotype pattern; CO840, representative strain of R3 ribotype pattern; DJ35, pre-cyclone isolate of *V. cholerae* O1; Cy30, post-cyclone isolate of *V. cholerae* O1.

these strains, as they might have a defective *rfb* gene with poor expression of O139 lipopolysaccharide or an altogether different serogroup that shares some homology with the O139 serogroup. Twelve *E. coli*



**Fig. 3.** (a) Comparison of the RAPD profiles of the O1 and O139 *V. cholerae* isolates from Orissa before and after the cyclone using primer 1281. (b) Comparison of the RAPD profiles of the O1 and O139 *V. cholerae* isolates from Orissa before and after the cyclone using primer 1283.

isolates were tested by PCR using different primers for the detection of virulent genes to detect different groups of diarrhoeagenic *E. coli*. One strain of *E. coli* gave the expected amplicon specific for EAaggEC (data not shown).

## DISCUSSION

Bacteriological and epidemiological investigations carried out in the cyclone-affected coastal saline tract of Orissa had revealed the prevalence of *V. cholerae* O1 among the hospitalized cases with acute diarrhoea. Sporadic cholera outbreak has been reported previously in Koraput and Nawarangpur district of Orissa due to *V. cholerae* O1 during 1993 [20]. Both the O1 and O139 serogroups of *V. cholerae* are involved in causing cholera outbreaks, in the cyclone-affected areas with predominance of O1 serogroup over O139 serogroup. Further, it was also found that all the *V. cholerae* O1 strains belong to biotype El Tor and serotype Ogawa, a common trend recorded in other cholera endemic regions [21]. It must be noted here that after the emergence of the *V. cholerae* O139 serogroup during 1992–3, the O1 Ogawa serogroup again became the only serogroup causing epidemic cholera [22].

All the *V. cholerae* O1 strains were sensitive to tetracycline, ciprofloxacin, chloramphenicol, norfloxacin and gentamicin; but were resistant to nalidixic acid, furazolidone, streptomycin, co-trimoxazole and ampicillin. A similar picture was observed among *V. cholerae* O1 isolated from Calcutta during and

after the emergence of the O139 serogroup [22]. Among the pre- and post-cyclone O139 strains, there was no considerable difference in antibiotic sensitivity pattern. The O1 and O139 isolated during the same time frame had an almost identical antibiogram with the exception of nalidixic acid.

Phenotypic characterization is less discriminatory in identifying the clonal nature of bacterial strains within a serogroup. To overcome this, many molecular techniques have been used recently to distinguish clones of enteric pathogens for epidemiological investigations. In this study, we have employed two techniques, namely RAPD and ribotyping, to identify and compare the clonality. Many investigations carried out in the past, indicated the existence of different ribotypes among *V. cholerae* O139 strains isolated from the same geographical locations [23–26]. Ribotypes of selected strains from cyclone affected areas exhibited only R3 type as has been reported from other parts of the country such as Calcutta, after the emergence of *V. cholerae* O139 during 1992–3 [26]. This suggests that no new ribotypes emerged among the El Tor biotypes of *V. cholerae* during the period under report. Further, the RAPD results exhibited similarity with Calcutta strains (*V. cholerae* O1 that appeared after the O139 Bengal epidemic). Similarly, *V. cholerae* O139 isolated in Orissa during the post-cyclone period showed similarity with SG 24, the strain isolated during 1992 cholera epidemic in Calcutta (unpublished data).

Even though *E. coli* was present in almost all the tested samples, we have given priority to the *E. coli*

strains that were isolated as a sole pathogen to screen for different virulence genes. This point was considered as the mixed infection of both toxigenic *V. cholerae* and the diarrhoeagenic *E. coli* is a very rare event during a cholera outbreak [27].

Due to invasion of seawater up to 30 km inland during cyclone period, followed by large number of cholera cases in these affected areas, it was presumed that there might be a new serogroup or at least new clones of *V. cholerae* involved in the outbreak as the marine milieu highly favours the vibrios for survival and proliferation [28]. However, in the present investigation all the clinical isolates of *V. cholerae* O1 and O139 exhibited similar clones as prevailing in the rest of the country. In addition, the clonal nature of *V. cholerae* O1 as well as O139 was identical to that of pre-cyclone isolates. Scarcity of drinking water and poor sanitation facilities would have been the main reasons for the cholera outbreak as has been observed in the past [29]. The analysis of epidemiological data collected from state Government, Health Department revealed that the case fatality rate due to diarrhoeal disorders was lower (0.08) as compared to the corresponding month of the previous year (7.8) due to the early detection and isolation of the organism and its quick reporting to initiate appropriate public health measures. Soon after the initiation of several control measures such as supplying drinking water through water tankers, oral rehydration salts (ORS), antibiotics and bleaching powder, the morbidity and mortality rate due to diarrhoea had been brought under total control in and around the super cyclone affected areas in Orissa.

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