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Incidence, Serotype, Antibiogram and Toxigenicity of *Vibrio cholerae* during 2000, Six Month after the Super Cyclone, 1999 in Orissa, India

Hemant Kumar Khuntia[1], Surya Kanta Samal[1], Sudeep Ranjan Nayak[2], Ashok Kumar Sarangi[3], Priyabrata Mohanty[4], Santanu Kumar Kar[1] and Bibhuti Bhusan Pal[1*]

[1]Department of Microbiology, Regional Medical Research Centre, Bhubaneswar - 751 023, India.
[2]Department of Microbiology, Allahabad Agriculture Institute (Deemed University), Allahabad, India.
[3]School of Biotech Sciences, TACT, Bhubaneswar - 751 024, India.
[4]Department of Botany, Utkal University, Bhubaneswar, India.

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This study was undertaken to analyze the cholera situation during July to November 2000 six month after the super-cyclone in saline tract of Orissa, India. Hundred ninety eight rectal swabs collected from hospitalized diarrhoea cases were subjected for bacteriological analysis. Of the 162 culture positive cases, *V.cholerae* 81 (50%), EPEC 1 (0.6 %), EHEC 3 (1.8%), EAggEC 2 (1.2%), *Shigella flexneri* 3 (1.8%) and Salmonella *spp.* 4 (2.5%) were isolated. Quadriplex and monoplex PCR assay revealed that, 51(31.5%) were *V. cholerae* O1 and 30 (18.5%) *V. cholerae* O139; carried *ctxA, tcpA* (Biotype El Tor), *zot, ace and toxR* except 3 *V. cholerae* O139 negative for *ctxA* gene. Incidence of *V. cholerae* six month after the super cyclone was found significantly higher than the pre-cyclonic period ($P < 0.5$). Strains of *V. cholerae* O1 was observed to be resistant to nalidixic acid, furazolidone, streptomycin, co-trimoxazole, ampicillin & neomycin. Except for co–trimoxazole, the resistant pattern of O139 was identical with that of O1 strains. The study revealed that *V.cholerae* O1 & O139 were the potential organism for cholera outbreaks in coastal Orissa, where *ctxA & tcpA* genes played a major role for pathogenesis. Incidence and emergence of fluroquinolone resistant *V. cholerae* O1 and O139 and nalidixic acid resistant O139 sero group should be closely monitored.

Key words: *Vibrio cholerae*, Quadriplex PCR, Diarrhoea, Antibiotic, Resistance.

In spite of improved methods of surveillance, diagnosis and treatment cholera still remains as an important cause of morbidity and mortality and possesses a major global public health problem. Global incidence of cholera with 94 countries reporting a high incidence of cholera cases to WHO[1] including India. According to the WHO estimate in 1998 a total 2, 93,111 cases of cholera and 10,586 deaths from it were reported[2]. The year 1990 have also witnessed an unprecedented emergence of new serogroup of *V.cholerae* O139 Bengal associated with epidemic cholera and subsequently the spread of new clone of both O1 and O139 serogroup to other parts of the country[3-6]. All these events made the etiologic

* To whom all correspondence should be addressed.
Tel.: +91-674-2302801(O)
E-mail: bbpal_rmrc@yahoo.co.in

role played by *V.cholerae* very complicated and warrant constant monitoring and systematic follow up.

Orissa, an eastern state of India inhabited by 3, 67, 06,920 number of people is situated from 17° 49' N to 22° 34 N latitude and from 81° 28 E to 87° 29 E longitude. Its long coastal saline tract has been experiencing frequent diarrhoeal out breaks every year. Both *V. cholerae* O1 and O139 have been isolated as etiological agent of such sporadic out break in the past[7,8] in Orissa. The state has also been subjected natural disaster like flood and cyclone quite often. In a recent episode of super cyclone in 1999, 9 districts of saline tract was affected and there was an outbreak of cholera due to *Vibrio cholerae* O1 and O139 having toxigenic genes[9].

The present study has been undertaken to investigate the cholera situation during the period July–November, 2000 after super cyclone in the coastal district of Orissa.

MATERIAL AND METHODS

Specimen collection

This was a part of the surveillance study conducted by Regional Medical Research Center (RMRC), Bhubaneswar between July-November, 2000 in 7 hospital of Puri and Cuttack districts in Orissa. Rectal swab samples were collected non-randomly twice a week from the hospitalized patients having acute diarrhoeal symptoms in the selected hospitals, before any antibiotics were administered. Diarrhea was defined as three or more watery or loose stools in a 24-hour period prior to admission to the hospitals. All the patients were interviewed using standard performa. A total number of 198 rectal swabs were transported to Microbiology and Pathology laboratory in RMRC in Carry Blair transport medium (CBT, Difco, USA) and were processed within 3-7 hours for the isolation of *V.cholerae* and other enteropathogens using standard techniques[10].

Bacteriological analysis

Bacteriological analysis of the rectal swab samples were carried out following standard methods. Rectal swabs were inoculated to MacConkey, Hekteon Enteric (Difco, USA) and thiosulphate-citrate-bile-sucrose Agar (Eiken, Tokyo, Japan) plates and kept at 37°C for 18-24 hours. Presumptive identification of *V.cholerae, E.coli, Shigella spp* and *Salmonella* spp were performed using previously published method[10]. Typical yellow colonies from TCBS colonies were inoculated into a multi-test medium for rapid presumptive identification of *V.cholerae*[11].

Polymerase chain reaction (PCR) assay

Template DNA was extracted from the culture grown in Luria Bertani (LB, Difco, USA) broth for overnight by boiling in a water bath for 10 min and cooling on ice immediately. A quadriplex PCR based assay was conducted to for simultaneous detection of genes specific for *V. cholerae* O1 and/or O139 serogroup (*wbe and/or wbf*), cholera toxin A-subunit (*ctxA*), toxin co-regulated pilus (*tcpA*) and central regulating protein ToxR (*toxR*) in a single tube reaction following method described elsewhere[12]. Monoplex PCR assay was performed for the detection of other toxic genes *zot* and *ace* present in the CTX genetic element region of the genome of *V.cholerae* following the method described earlier[13]. Briefly, the optimized quadriplex PCR protocol was carried out with 35 ìl reaction mixture which contained 10x amplification buffer [100mM Tris (P^H-9.0), 500 mM KCl, 0.1% Gelatin] (Bangalore Genei, India); 2.5 ìl Magnesium chloride (25mM); 2.5 ìl each of 2.5mM dATP, dCTP, dGTP and dTTP (Bangalore Genei, India), 90 pmol each *tcpA* (El Tor) and *tcpA* (Classical), 68pmol each *ctxA*, *wbe* O1, *wbf* O139 and 60 pmol *toxR*, 1.2U of *Taq* DNA-polymerase (Bangalore Genei, India) and Milli-Q water to a final volume of 29.5 ìl and 5.5 ìl cell lysate (Template DNA). Finally, the reaction mixture was over laid with a drop of sterile mineral oil (Bangalore Genei, India). Amplification was carried out as follows, 4 minute at 94°C for initial denaturation followed by 30 cycles of 1.5 minute at 94°C, 1.5 minute at 55°C and 1.5 minute at 72°C with a final round of 7 minute at 72°C in a thermal cycler (Techne, England). PCR product (12 ml) was visualized by UV trans-illuminator after electrophoresis in 2% agarose gels in Tris-borate-EDTA buffer at 100V for 45min and ethidium bromide staining (0.5ìg/ml).

Antimicrobial susceptibility

The sensitivity and resistant pattern for both O1 and O139 strains were examined by using

Table 1. Distribution of *V. cholerae* among age, sex and geographical in the student area

Hospital	Rectal swabs			Age group		*Vibrio cholerae*				*V. cholerae* Male/ Female	*V. cholerae* (%), among diarrhoea cases
	Male	Female	Total	Pediatric No. of *V. cholerae* / Total	Adult	O1 M / F	O1 Total	O139 M / F	O139 Total		
Satyabadi	35	25	60	4 / 37	33 / 37	16 / 14	30	5 / 2	7	21 / 16	37 (61.7)
Balanga	18	25	43	8 / 15	7 / 15	8 / 6	14	1 / 0	1	9 / 6	15 (34.9)
Kakatpur	8	4	12	0 / 5	5 / 5	1 / 0	1	3 / 1	4	4 / 1	5 (41.7)
Nimapara	6	5	11	2 / 4	2 / 4	0 / 0	0	1 / 1	4	2 / 2	4 (36.4)
Astaranga	6	0	6	0 / 2	2 /2	0 / 0	0	1 / 1	2	1 / 1	2 (33.3)
Pipili	5	17	22	1 / 4	3 / 4	0 / 3	3	1 / 0	1	1 / 3	4 (18.18)
Rushipada	8	9	17	0 / 10	10 / 10	0 / 1	1	4 / 5	9	4 / 6	10 (58.8)
Jagatsinghpur	7	2	9	0 / 1	1 / 1	1 / 0	1	0 / 0	0	1 / 0	1 (11.1)
Balikuda	2	3	5	0 / 1	1 / 1	0 / 1	1	0 / 0	0	0 / 1	1 (20.0)
Kendrapara	2	2	4	0 / 2	2 / 2	0 / 0	0	2 / 0	2	2 / 0	2 (50.0)
Ersoma	6	3	9	0 / 0	0 /0	0 / 0	00	0 / 0	0	0 / 0	0 (0.00)
Total (%)	103 (52)	95 (58)	198	15 / 81 (18.5)	66 / 81 (81.5)	26 / 25 (51) / (49)	51	19 / 11 (63.3)/(36.7)	30	45 / 36 (55.5) / (44.5)	81 (40.9)

where as O139 were isolated more from males (63.3%) than the females (36.7%) (Table 1). The adults were more affected 66 (81.5%) by cholera than the pediatric age group 15(18.5%). More number of rectal swabs 60 (30.3%) and *V. cholerae* 37 (45.6%) was isolated from diarrhoea patients in Satyabadi hospital in comparison to other studied hospital which indicates that population under this hospital was severely affected by cholera outbreaks. Highest 103 (52%) diarrhoea cases and 56 (69.1%) *V. cholerae* (O1 and O139) were isolated in the month of September which constitute the peak period of the outbreak during 2000. Resistance pattern of O1 and O139 were CoFrNaNS and AFrNSNa respectively. Both serogroups were sensitive to CfNxCTG while O1 was resistant and O139 was sensitive to co-trimoxazole. Ciprofloxacin (39%), norfloxacin (12%) resistant *V.cholerae* O1; ciprofloxacin (33%), norfloxacin (9.6%) and nalidixic acid (100%) resistant *V. cholerae* O139 were encountered during the study period.

Quadriplex and monoplex PCR analysis performed on all the isolated strains of O1 and O139 revealed that all the tested strains were positive for *ctxA*, *tcpA* (Biotype El Tor), *toxR* (top regulating), *zot* and *ace* genes except 3 *V. cholerae* O139 strains which did not amplified *ctxA* gene. There exists several undocumented past history of diarrhoeal diseases with large morbidity and mortality in Orissa. However for the first time during 1993, *V. cholerae* O1 was isolated as epidemic strain from an outbreak in Nawarangpur, Koraput district[7]. Then there was an emergence of O139 during 1995 outbreak in Orissa[8]. During 1999 super cyclone, both O1 and O139 serogroups were involved causing cholera outbreaks where O1 was the dominant serogroup[9]. Although *V. cholerae* O1 and O139 serogroups were responsible as the major etiological pathogen causing diarrhoea outbreaks, O1dominanted O139 serogroup. But it was observed that there was an increasing trend of occurrence of O139 serogroup (37%) during the present study in comparison to that of during 1999(9%) outbreak.

It has been reported that usually one of the two serotypes is responsible for majority of cholera outbreak in any geographical area, but one serotype replaces the other with time in an endemic area[16,17]. This evidence has been witnessed by the emergence of novel strain of *V. cholerae* O139 as epidemic strain during 1992-93[4], the return of O1 during 1994-1996 as dominant serogroup[18] and resurgence of *V.cholerae* O139 during 1996-97[19]. This change in sero-dominancy has been attributed to be correlated with immune status of the population and has been documented in animal models[20]. Therefore it can not be ruled out from the trend of the present study that O139 may outnumber O1 and become the dominant serogroup in forth coming years much in the same fashion like the resurgence of O139 during 19996-97 in Calcutta[19]. Several instances of unpredictable appearance, disappearance, and reappearance of multiple drugs including tetracycline, co-trimoxazole, neomycin resistant *V. cholerae* strains have been encountered in the history of cholera[21-24]. On the contrary in October 1995 nalidixic acid resistant *V. cholerae* O1 was encountered in South India[25], while our study revealed the emergence of fluroquinolones resistant *V. cholerae* O1 and O139 during 2000 and complete resistance of O139 to nalidixic acid for the first time in Orissa. The variability of the resistance pattern of O139 for nalidixic acid in different time period and different geographical region is a matter of discussion. It was found that O139 strains isolated during 2000 from Calcutta outbreak were sensitive to nalidixic acid while the same serogroup from other outbreak areas were resistant[26]. In this context Orissa all the O139 strains isolated during 2000 were resistant to nalidixic acid while the O139 isolates of earlier years were sensitive[8, 10]. Our earlier study exhibited that the clonality of O139 of 1999 were similar to SG24; the strains isolated in Calcutta during O139 epidemic in 1992[9]. The O139 strains isolated in the same cyclone area during 2000 with changing sensitive patterns to the nalidixic acid, have received considerable attention and create a question about the clonal change as has been seen in resurgence of *V. cholerae* O139 during 1996-97 in Culcatta with altered antibiogram pattern leading to an unique change in structure and organization of CTX Φ module[27].

The toxigenic status of both O1 and O139 was determined by recognizing the presence of *ctxA*, *tcpA, ace* and *zot* genes by PCR assay,

suggesting that *ctxA* gene plays a major role for pathogenesis of diarrhoea in these outbreaks. However, three clinical O139 isolates negative for *ctxA* gene carrying *zot* and *ace* gene might have caused watery stool due to ACE and ZOT toxin. The association of 6 diarrhoeagenic *E.coli* suggests that the rest 55 *E.coli* could be commensal organism. Even though 6 number of *E. coli* were isolated in the present study as sole pathogen, the point of mixed infection of both toxigenic *V. cholerae* and diarrhoeagenic *E. coli* was considered and ruled out as is a very rare event during outbreak.

Although sample collection and isolation for *V. cholerae* began from first week of July, the rising trend started from 2nd week of September and reached its peak by the 4th week of the same month and thereafter continued to decrease up to 2nd November. The population under Balanga hospital, Satyabadi PHC and ID hospital, Puri were severely affected by cholera outbreak; which were less affected by super cyclone in 1999. In contrast, the earlier study had reported November 1999 as the peak after super cyclone which occurred due to the favorable marine milieu due to the invasion of sea water during the super cyclone. The significant increase in the incidence of *V. cholerae* after six month of the super cyclone in comparison to the pre-cyclonic incidence ($P < 0.5$) may be due to the optimum favorable condition produced in aquatic environment by super-cyclone which better suited the *V. cholerae* for survival during inter-epidemic period and subsequently started several onset of outbreaks during post monsoon period.

CONCLUSION

The increase of occurrences of several cholera outbreaks during aftermath period of the super cyclone is note worthy in the present investigation. The incidence of *V. cholerae* O1 Ogawa, El Tor biotype was the dominant serogroup, while *V. cholerae* O139 appeared with increasing trend coupled with altered antibiogram. Emergence of fluroquinolones resistant *V. cholerae* should be carefully monitored. Scarcity of safe drinking water and sanitary facilities has been stressed as the main cause of cholera outbreak in Orissa in the past. Poor socioeconomic condition population explosion coupled with lack of hygiene; further aggravates the cause. However, continuous surveillance and early diagnosis coupled with better standard of sanitation and personal hygiene can significantly limit the spread of infection and minimize the public health problem.

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