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Original Research Article

Molecular characterization of Vibrio cholerae O1 in Hyderabad

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Abstract

Cholera has been the subject of numerous investigations from both bacteriological and epidemiological points of view. The seventh pandemic witnessed emergence of several new types of Vibrio cholerae. Further new variants and hybrids of Vibrio cholerae O1 Eltor have been reported in the recent past. One hundred eighty five stool specimens, 100 from sporadic cases and 85 from outbreak collected from clinically suspected cases of cholera in Bholakhpur, Andhra Pradesh, were subjected to culture, antibiogram by disk diffusion method, serotyping, phage typing, and Mismatched Amplification Mutation Assay (MAMA PCR). Out of 100 sporadic samples processed, 39 were culture positive for Vibrio cholerae and of the 85 outbreak samples 22 were culture positive. This study reports the isolation of variant of Vibrio cholerae O1 Eltor carrying classical ctx B gene for the first time in Andhra Pradesh.

Keywords: Biotype; Escherichia coli; Phage type; Serotype; Vibrio cholerae

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1. Introduction

Cholera has been the subject of numerous investigations from both bacteriological and epidemiological points of view. It presents a constant challenge to the bacteriologist and epidemiologist alike. Six pandemics of cholera were caused by Vibrio cholerae O1 classical biotype. The seventh pandemic has witnessed the emergence of several new types of Vibrio cholera including the Eltor biotype and the toxigenic non O1 serogroup O139. Further new variants and hybrids of Vibrio cholerae O1 Eltor has been reported in the recent past. The classical and Eltor biotypes are closely related in their O antigen biosynthetic gene but differ in the CTXφ genome which is responsible for cholera toxin CTXφ class is found in classical strains, CTXφ ET is found in Eltor and O139 strains and CTXφ Calcutta is found in resurgent Eltor strains.[1] Methods for differentiating the biotype specific ctxB subunit of Vibrio cholerae O1 include, sequencing of ctxB gene, monoclonal antibody based serology and mismatched amplification mutation assay (MAMA PCR), this assay detects sequence polymorphism at nucleotide position 203 of ctxB gene.[2] In this study we report the isolation of variant of O1 Eltor carrying ctxB gene.

2. Materials and methods

A prospective study was conducted during the period September 2008 to August 2009 in Bholakhpur, Andhra Pradesh. During this study period 185 clinically suspected cases of cholera (100 samples from sporadic cases and 85 samples from outbreak occurred during the month of May at Bholakhpur) were included. Specimens were collected in sterile bottles before starting antibiotics and transported to the laboratory for processing. Specimens were cultured directly on MacConkey's agar, Nutrient agar and thiosulphate citrate bile salt sucrose agar (TCBS) agar, xylose lysine deoxycholate agar (XLD agar). In addition to direct plating specimens were subcultured on MacConkey's agar, TCBS agar after enrichment in alkaline peptone water. Plates were examined after overnight incubation at 37°C. Colonies suggestive of Vibrio cholerae were identified by standard biochemical tests[3] and confirmed by serotyping using high titre sera obtained from King Institute of Preventive Medicine, Guindy, Chennai. O385 (classical) and N16961 (Eltor) control strains were used. Biotyping was done by conventional methods such as chick cell agglutination, VP test, Polymyxin B sensitivity, sheep RBC haemolysis[3]. Phage typing by new and old methods and susceptibility to phage IV and phage V for biotyping was done at Vibrio Phage Reference Laboratory, National Institute of Cholera and Enteric Diseases (NICED), Kolkata. Mismatched Amplification Mutation Assay PCR (MAMA PCR) for differentiation of ctxB classical and ctxB Eltor was done at bacteriological division of NICED (National Institute of Cholera and Enteric Diseases). E. coli isolates from 11 outbreak stool samples from children under 5 years of age group, 3 colonies from each sample were sent to NICED. Diarrhoegenic E.coli was identified by multiplex PCR. Water samples collected from household municipal taps, stored water in the houses and reservoir of the area were bacteriologically analyzed by the Institute of Preventive Medicine (IPM) during and preceding the outbreak. Antibiotic susceptibility testing was performed by Kirby Bauer's method. The following commercial (Hi Media) antibiotic disks were used Tetracycline (30µg), Ampicillin (10µg), Ciprofloxacin (5µg), Cotrimoxazole (25µg), Amikacin (30µg) and Cefotaxime (30µg). The plates were read after 16-18 hours incubation at 37°C. The zone of inhibition for each antibiotic was interpreted as per Clinical and labortory standards guidelines (CLS).

3. Results

One hundred eighty five stool samples were collected from clinically suspected cases of cholera, 100 from sporadic cases and 85 from outbreak during the period September 2008 to August 2009. The outbreak started on 3rd May 2009 as common source outbreak with admission of 8 children. Adults presented later on 5th May 2009. Acute rise in cases, with maximum number of cases were admitted on 6th May 2009 with a fall on 11th May 2009 and ended on 25th May 2009. Males were more susceptible among both the sporadic and outbreak cases (Fig. 1). The predominant age group affected among sporadic cases was the age group between 15-25 while two peaks were observed among outbreak cases one was under 4 years group and the other among age group between 15-25 (Fig. 2). None of the sporadic cases developed complications. Complications like severe dehydration, fever, renal failure were seen among outbreak cases. Mortality rate was nil among sporadic cases while it was 0.22% during the outbreak with one death each among children and adults.

One hundred sporadic samples and 85 outbreak samples were processed. Out of 100 sporadic samples processed, 39 were culture positive for Vibrio cholerae and of the 85 outbreak samples 22 were culture positive (Table 1). Vibrio cholerae was the major isolate followed by E. coli both among sporadic and outbreak cases (Table 2). All the 61 Vibrio cholerae positive samples (39 of sporadic cases and 22 of outbreak cases)

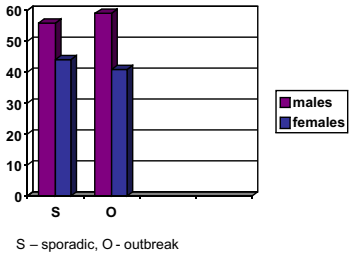


Fig. 1: Sex wise distribution of cases

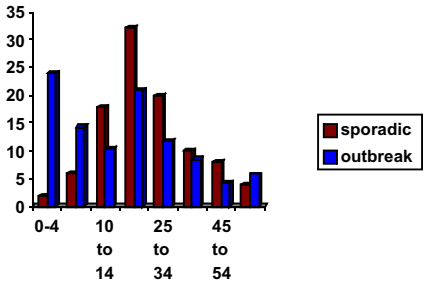


Fig. 2: Age wise distribution of cases

Table 1: Rate of isolation of Vibrio cholerae

Type of strain	No. of cases	Culture positivity		Culture negativity	
		No.	Percentage	No.	Percentage
Sporadic	100	39	39	61	61
Outbreak	85	22	25.8	63	75.1

belonged to serogroup O1, Biotype - Eltor, serotype - Ogawa. Eleven E.coli isolates from children less than 5 years were sent to NICED for pathotyping, of which 2 were found to be enterotoxigenic type and 1 was entero aggregative type. Water analysis report from IPM also showed faecal contamination of water due to E. coli. As there was sewage contamination, multiple organisms were isolated.

Phage typing was done at Vibrio Phage Reference Laboratory, Kolkata. A total of 20 outbreak strains were phage typed. Phage typing was done by Basu and Mukherjee method and new phage typing method. Ninety nine percent of sporadic strains were typable while only 60% of outbreak strains were typable and 40% were untypable. T2 was the predominant phage type (Table 3). Ninety nine percent of sporadic strains were typable. Even with the new method 40% of outbreak strains were untypable. Among the typable strains, T27 was the predominant phage type and other phage types, i.e T13, T7 were found only among outbreak strains (Table 4). All the strains showed uniform sensitivity to Amikacin, Cefotaxime, Ciprofloxacin and 100% resistance to Cotrimoxazole and Amoxicillin (Fig. 3). Through MAMA PCR, 60 out of 61 strains were toxigenic and all toxigenic Eltor strains carried classical toxin gene.

Table 2: Rate of isolation of enteric pathogens

Pathogen	Sporadic			Outbreak		
	No.	No. of positive strains	Percentage	No.	No. of positive strains	Percentage
Vibrio cholerae	100	39	39	85	22	25.8
Escherichia coli	100	17	17	85	11	12.9
Shigella	100	1	1	85	--	--
Campylobacter	100	--	--	15	2 out of 4*	13.3

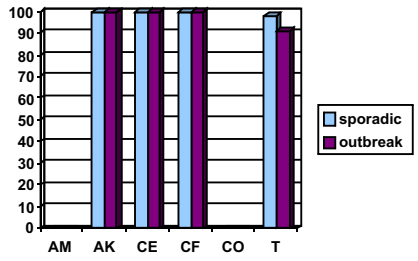
*4 specimens were processed (at NICED) out of which 2 were positive for Campylobacter.

Table 3: Basu and Mukherjee phage typing method

Phage type	Sporadic		Outbreak	
	No.	Percentage	No.	Percentage
T2	38	99	12	60
UT	1	1	8	40
Total	39	100	20	100

Table 4: New phage typing method

Phage type	Sporadic		Outbreak	
	No.	Percentage	No.	Percentage
T27	38	99	6	30
T13	-	-	5	25
T7	-	-	1	5
UT	1	1	8	40
Total	39	100	20	100



AM-amoxycillin,AK – amikacin, CE - cefotaxime, CO – cotrimoxazole, T – tetracycline.

Fig. 3: Antibiotic sensitivity pattern

4. Discussion

Annual outbreaks of cholera are a regular feature in our country. A high population density along with open drains and poor sanitation provides an optimal niche for survival, sustenance and transmission of Vibrio cholerae. The seasonal outbreaks are reminder of endemicity of the illness and its emergence as an important pathogen of acute watery diarrhea.[4] More discriminatory procedures and molecular biology techniques are now assisting the epidemiologists in understanding the evolution and spread of Vibrio cholerae clones and emergence of new variants.[5]

In this study all the 61 Vibrio cholerae isolates belonged to serogroup O1, Eltor biotype and Ogawa serotype. Eltor biotype was reported for the first time from Andhra Pradesh in Visakhapatnam in the year 1965 by Bhaskaran and spread to other parts of the state and completely replaced classical biotype in the latter half of 1965.[6] O139 was reported for the first time

in Chennai and spread to various other places. However O139 was reported from Hyderabad in 1993. It continued to coexist with Eltor till 2004 and in 2005, Eltor completely replaced O139. In our study O139 was not detected. Sixty one vibrio cholerae strains were subjected to MAMA PCR, except for one strain all were found to be toxigenic and all the toxigenic strains carried classical toxin gene (ctx B). Since these strains phenotypically exhibited Eltor characteristics and genotypically showed classical toxin these were categorized as Eltor variants. Eltor variants were reported for the first time from Bangladesh in 2002.[7] Analysis of Vibrio cholerae strains isolated in Kolkata from 1989-2005 showed the appearance of Eltor variants in 1995 and total replacement of Eltor biotype in 1999.[8] A study conducted in Chandigarh in 2005 showed that 80% of the Vibrio cholerae strains isolated were found to harbor both ctxB of classical Eltor genes.[9] Molecular characterization of the cholera ctx gene and the Vibrio pathogenicity island (VPI) shall be useful in tracking the spread of the new variants in monitoring further changes in the genome of Vibrio cholerae as its genome seems to be in fluid state and the changing climate and ecosystem can bring almost many more changes.

Prevalent serotypes have varied from year to year. Ogawa was predominant in early 1970's whereas in 1975 Inaba was predominant. During the past 14 years, Ogawa has become the prevalent serotype except for few reports from Delhi. While serotype Inaba appeared in Hyderabad during the year 2005-2007, in the present study from 2008-2009 only serotype Ogawa was isolated. Though 80% of the strains were of serotype Inaba in 2007, 2008 has seen abrupt replacement by Ogawa serotype.[10] Such a change in serotype patterns was also reported from a study conducted in Sevagram and found that until 1999, Ogawa was the prevalent serotype which was then replaced by Inaba.[5] Similar shifts in serotypes were also reported from Kolkata and Delhi. Phage typing for Vibrio cholerae is one of the best established tool and marker for epidemiological characterization of the isolates.[5] Phage typing was done by old and new methods. Basu and Mukherjee classified into 6 groups using 5 phages. According to new typing method 100% of the strains are typable.

According to old phage typing method our study showed T2 as the predominant phage type which correlated with various other studies.[10-11] Some studies conducted in India reported T4 as the predominant phage type.[12-14] A few studies from South India reported T2 and T4 phage types. [16,17] With the new phage typing method our study showed T27 as the predominant phage type which correlated with various other studies.[10-17] In our study other phage types i.e. T13 and T7 were seen only among outbreak strains which was in contrast to other studies which reported T26,T23,T21,T25 phage types.[12,14,17]

A variation in phage typing was observed among sporadic and outbreak strains. Ninety nine percent of sporadic strains were typable while only 60% of the outbreak strains were typable. Among the typable strains T27 was the predominant phage type among both sporadic and outbreak strains. T13 and T7 were the other phage types seen among outbreak strains which were not seen among sporadic strains. Fourty percent of the outbreak strains were not typable even with the new method which indicates some new strains might have emerged which lead to this outbreak. This also underscores the need for more discriminatory methods. There was high degree of resistance to cotrimoxazole (100%) and amoxicillin (100%) exhibited by Vibrio cholerae in our study. A study conducted in Delhi also showed similar findings, while a study from Maharashtra reported 65% sensitivity to Cotrimoxazole.[4,5]

5. Conclusion

All Vibrio cholerae strains isolated in the present study were found to be Eltor variants, reported for the first time from Andhra Pradesh. All strains

showed 100% resistance to cotrimoxazole. Fourty percent of the outbreak strains were untypable even with the new phage typing method. Phenotypic and genotypic characterization showed similarities among sporadic and outbreak strains, but variation was observed among the phage types which showed that some new strains have emerged during the outbreak. Though Eltor biotype was replaced by Eltor variant in Hyderabad, continuous monitoring of the strains from other parts of the state and country is required to know whether Eltor biotype has been completely replaced by Eltor variant.

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