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Tetracycline Resistant Vibrio Cholerae O1 Ogawa With Ctxb7 Allele (Haitian) is Prevalent in Haryana, India

KEYWORDS: V. cholerae O1, DMAMA PCR, ctxB, Haitian genotype, Antibiotic resistance

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ABSTRACT *Assessment of antimicrobial resistance and cryptic changes in the ctxB gene in Vibrio cholerae O1 are important for the surveillance and better management of cholera disease. The present study was undertaken to assess the resistance of tetracycline and variation in ctxB of V. cholerae O1 isolated from different parts of Haryana. Bacterial characterisation was done using biochemical and serological assays. Double mismatch amplification mutation assay (DMAMA) PCR was used for the detection of ctxB7 (Haitian genotype). All isolates were identified as V. cholerae O1 biotype El Tor serotype Ogawa. DMAMA PCR assays confirmed the presence of ctxB7 Haitian genotype in all the isolates. These isolates were multidrug resistant including tetracycline. V. cholerae isolates were commonly resistant to nalidixic acid, co-trimoxazole and tetracycline in this region and a new variant of ctxB was also identified.*

Introduction

The acute diarrheal disease cholera is a major public health problem in Asia, Africa and Latin America (Farque, SM *et al.*, 2002). Cholera is caused by ingestion of food or water contaminated with the gram-negative bacterium *Vibrio cholerae*. In endemic countries, about 91,000 people die of cholera among the 2.8 million cholera cases that occur annually (Ali, M *et al.*, 2012). This disease is typically characterised by frequent passage of rice watery stools. The other clinical manifestations of cholera include symptoms such as abdominal cramps, vomiting and severe dehydration (Weir, E *et al.*, 2004). Death may result if the patients with these symptoms are not treated properly. *V. cholerae* has more than 206 known serogroups of which only O1 and O139 can cause epidemic and pandemic cholera (Faruque, SM *et al.*, 1998). The serogroup O1 is classified into two biotypes, which are termed 'classical' and 'El Tor'. The current seventh pandemic of cholera is caused by the El Tor biotype and the classical biotype of *V. cholerae* O1 is thought to have become extinct with the emergence of El Tor biotype. Indeed, the classical biotype has not featured in epidemic cholera since 1983 (Samadi, AR *et al.*, 1983). Another serogroup called *V. cholerae* O139 synonym Bengal was first discovered in 1992 when it caused several outbreaks in India and Bangladesh (ICDDRB, 1993, Ramamurthy, T *et al.*, 1993). A devastating cholera outbreak, that has occurred in Haiti since October 2010, has reported involving 6,98,893 cases and 8,540 deaths (UN FACT SHEET, 2014). Based on the mutations in the *ctxB* gene that encode cholera toxin B-subunit, the El Tor vibrios are classified into several toxin genotypes (Goel, AK *et al.*, 2008, Naha, A *et al.*, 2012). *V. cholerae* strains harboring *ctxB* genotype 7 (Haitian genotype) has been reported among strains in Haiti, Cameroon and Nepal (Hendriksen, RS et al., 2011, Talkington, D *et al.*, 2011). *V. cholerae* isolates having this CT genotype has prevailed in Kolkata since 2006 (Naha, A *et al.*, 2012, Kutar, BMRNS et al., 2013) and caused a large cholera outbreak in Orissa in 2007 (Goel, AK *et al.*, 2008).

Rehydration is the best way of treatment of cholera, which include oral rehydration solution (ORS) or intravenous fluids (IVF) with electrolytes (Brunkard, JM *et al.*, 2014). As a supportive therapy, antibiotics are also in use, which can reduce fluid requirements and duration of illness (Sack, DA *et al.*, 2004).Tetracycline is the primary antibiotic for the treatment of cholera in adults, whereas, furazolidone or co-trimoxazole has been recommended for children less than 9 years (ODH- IDCM Revised 1, 2014). During 1977, emergence of multidrug resistant *V. cholerae* El Tor was first reported in Tanzania (Mhalu, FS *et al.*, 1979). Tetracycline resistance in *V. cholerae* is plasmid mediated and these mobile genetic elements are not stable and hence the resistance patterns fluctuate in this organism (Taneja, N *et al.*, 2010). Other genetic elements such as efflux pumps, chromosomal mutations, conjugative transposons, integrons or self transmissible chromosomally integrating SXT elements are responsible for antibiotic resistance in *V. cholerae* (Kitaoka, M *et al.*, 2011). In several cholera-endemic countries, resistance to ampicillin, kanamycin, sulphonamides, streptomycin, co-trimoxazole, norfloxacin, gentamicin, furazolidone, ciprofloxacin, erythromycin, nalidixic acid and doxycycline have been reported (Faruque, ASG *et al.*,2007, Sack, DA *et al.*, 2004, Kitaoka, M *et al.*, 2011). In this study, we focused our interest on changing antimicrobial resistance patterns and the type of CT genotype existing among *V. cholerae* O1 isolated from the patients referred from different parts of Haryana and admitted in Maharishi Valmiki Infectious Diseases Hospital (MVIDH), Delhi.

Materials and Methods

Collection and processing of samples

Rectal swabs were collected at the time of admission from all the 31 suspected cholera or acute gastroenteritis patients from different parts of Haryana referred to MVIDH,

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Delhi in 2012. The samples were transferred to alkaline peptone water (APW, pH-8.6) and incubated at 37°C for 4-6 hrs. After incubation, the enriched cultures were further inoculated to the thiosulphate-citrate-bile-salts-sucrose (TCBS) agar and bile salt agar (BSA, pH-8.6) (Hi-Media, Mumbai) (Sharma, NC *et al.*, 2007).

Confirmation of V. cholerae

Typical colonies appearing on TCBS/BSA were confirmed by standard biochemical tests (CDC/WHO/USAID USA, 2003).These isolates were further tested serologically (Kay, BA *et al.*, 1994) with commercially available *V. cholerae* O1 polyvalent and monovalent and O139 antiserum (BD, USA and Denka Seiken, Ltd. Japan).

Antibiotic susceptibility testing

Antimicrobial susceptibility test was carried out by disk diffusion methods (Bauer, AW *et al.*, 1996, CLSI., 2013) using commercially available disks (BD, Difco, USA) of ampicillin (AM-10µg), azithromycin (AZM-15µg), cefotaxime (CTX-30µg), ceftriaxone (CRO-30µg), chloramphenicol (C-30µg), ciprofloxacin (CIP-5µg), co-trimoxazole (COT-25µg), gentamicin (GEN-10µg), imipenem (IPM-10µg), nalidixic acid (NA-30µg), norfloxacin (NX-10µg), ofloxacin (OF-5µg) and tetracycline (TE-30µg) using Mueller-Hinton agar (Hi-Media). *Escherichia coli* ATCC 25922 strain was used as a quality control strain. Results were recorded as resistant, reduced susceptible and sensitive as per guidelines of Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2013).The Minimum inhibitory concentration of tetracycline was determined for all tetracycline resistant isolates by E-test (AB Biodisk, Solna, Sweden). In this study, isolates showing reduced susceptibility were considered as resistant strains.

Genomic DNA preparation

Overnight culture from Luria-Bertani agar (LA) plate was suspended in 200 µl of TE buffer. To this suspension, 150 µl of phenol: chloroform: isoamylalcohol (25:24:1) was added and mixed by vortexing followed by centrifugation at 12,000 rpm for 10 mints. The supernatant from upper layer was taken and 200 µl of chloroform: isoamylalcohol (24:1) was added and mixed by vortexing for 5 mints. This mixture was centrifuged at 12,000 rpm for 15 mins and the supernatant containing genomic DNA was separated into a fresh microfuge tube. Dilutions of genomic DNA were made using sterile distilled water to obtain a concentration of 100ng/µl.

PCR assays for detection of *ctxB* genotype

Double mismatch amplification mutation assay (DMAMA) PCR was recently developed to differentiate the *ctxB* of classical (genotype 1), El Tor (genotype 3) and Haitian (genotype 7) by substitution in nucleotide on positions 58 and 203 of the *ctxB* gene (Naha, A. *et al.*, 2012). DMAMA PCR was used in this study to detect the *ctxB* genotype using primer pair *ctxB-F3* with *Rv-Cla* and *ctxB-F4* with *Rv-Cla* as described previously (Naha, A *et al.*, 2012, Morita, M *et al.*, 2008). *V. cholera*e O1 strains 569B (classical), N16961 (El Tor) and DL32521 (Haitian) were used as control strains. The primers used were synthesized by Invitrogen, India and dNTPs, Taq polymerase, 10X Taq buffer used in this study were obtained from Genei, Bangalore.

Results

Characterisation of isolates

A total of 12 *V. cholerae* isolates were obtained from 31 clinical samples in 2012 from different parts of Haryana (Table 1). All isolates were biochemically identified as *V. cholerae* biotype El Tor and serologically confirmed with polyvalent O1 and then agglutination with monovalent Ogawa.

Antibiotic susceptibility and MIC testing

All 12 *V. cholerae* O1 Ogawa isolates were found to be 100% resistant to nalidixic acid followed by co-trimoxazole (91.7%), ampicillin (66.7%), tetracycline (41.7 %), ceftriaxone (30%) and cephotaxime (25%) (Table-2). However, all the strains were susceptible for azithromycin, chloramphenicol, ciprofloxacin, gentamicin, imipenem, norfloxacin and ofloxacin (Table-2). The MIC of tetracycline in resistant strains was 8µg/ml, which is in the reduced susceptibility range as per the CLSI guidelines (2013).

Confirmation of *ctxB* gene by DMAMA PCR

All *V. cholerae* O1 strains were found to have *ctxB* of Haitian genotype *(ctxB*7) (Fig. 1) without any amplified PCR product of classical genotype (Fig. 2).

Discussion

Some areas of Haryana are endemic for cholera (Sharma, NC *et al.*, 2007, Taneja, N *et al.*, 2010) and suspected cholera cases are referred to MVIDH, Delhi from different parts including the cases from Gurgaon and Faridabad (Table1). As per our previous investigation these regions seems to be endemic for cholera (Sharma, NC *et al.*, 2007). There was one family outbreak consisting of six patients from Mewat (Unpublished data). Out of six, three were positive for *V. cholerae* O1 Ogawa belonging to genotype 7. In 2007, a genetic modification in the *ctxB* gene by substitution of amino acid histidine (H) at Position 58(C→A) was seen, which resulted in an amino acid substitution from histidine (H) to asparagines (N) at position (20) (genotype 7) in a large cholera outbreak in Orissa (Kumar, P *et al.*, 2009). This amino acid substitution has been found in Haitian outbreak strains from 2010. Haitian genotype (*ctxB*7) has been circulating in several regions of Haryana and to our knowledge this is the first report, indicating that strains of *ctxB* genotype 7 have spread beyond Orissa and Kolkata (Kumar, P *et al.*, 2009, Naha, A *et al.*, 2012, Kutar, BMRNS et al., 2013).

V. cholerae O1 Ogawa isolates were found to be multidrug resistant including tetracycline, a drug of choice for cholera treatment (WHO, 2005). Tetracycline resistant *V. cholerae* O1 was also reported from an epidemic of cholera in Tanzania in 1977-78 due to over use of this drug (Mhalu, FS *et al.*, 1979). Tetracycline resistant *V. cholerae* O1 has been reported from different parts of India, including Delhi (Sharma, NC *et al.*, 2007, Das, S *et al.*, 2011). In 2007, tetracycline resistant *V. cholerae* O1 has emerged in Kolkata with 76% isolates resistant to this drug as against 1% in 2004 (Bhattacharya, K *et al.*, 2011). In 2008, tetracycline resistant *V. cholerae* O1 biotype El Tor serotype Ogawa (67%) was reported from Chandigarh (Taneja, N *et al.*, 2010). Tetracycline resistant *V. cholerae* O1 has also been reported from different parts of major cholera epidemic countries like Latin America, Tanzania, Bangladesh and Zaire (Garg, P *et al.*, 2000). In present study we found tetracycline resistant in 42% of *V. cholerae* O1 Ogawa circulating in different areas of Haryana. Our finding suggests that tetracycline has been extensively used in these areas. The MIC of tetracycline has been reported ranging between 8µg/ml to 32µg/ml from different parts of the country (Bhattacharya, K *et al.*, 2011, Bhattacharya, D *et al.*, 2012, Das, S *et al.*, 2011, Mandal, J *et al.*, 2012, Taneja, N *et al.*, 2010) but we have noticed that the MIC was 8µg/ml in all tetracycline resistant strains. All strains were sensitive to azithromycin, chloramphenicol, ciprofloxacin,

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gentamicin, imipenem, norfloxacin and ofloxacin. Based on our finding, azithromycin or fluoroquinolones are the other choices in for cholera if there is a treatment failure due to tetracycline resistance.

Conclusion

This study has revealed the spread of Haitian genotype in above mentioned areas of Haryana for the first time to our knowledge. Hence the monitoring of spread of Haitian genotype is important since the morbidity is higher than the one caused by the El Tor biotype. Multidrug resistant *V. cholerae* O1 strains are circulating in this region, especially resistant to tetracycline, and hence empirical therapy to this drug should also be monitored carefully.

Figure Legend

Fig. 1: 2% gel Electrophoresis shown *ctxB-F3* with *Rv-Cla* for detecting the *ctxB* of Haitian genotype. Lane M- 100 bp molecular weight marker, lane 1- N16961 El Tor control strain showing negative band, lane 2- 569B Classical control strain showing negative band, lane 3- DL32521 Haitian control strain yielded 191 bp product, lane 4- Negative control, lane 5- HR32285, lane 6- HR32286, lane7- HR32289, lane 8- HR32355, lane 9- HR32626 and lane 10- HR33102 yielded 191 bp product.

Figure Legend

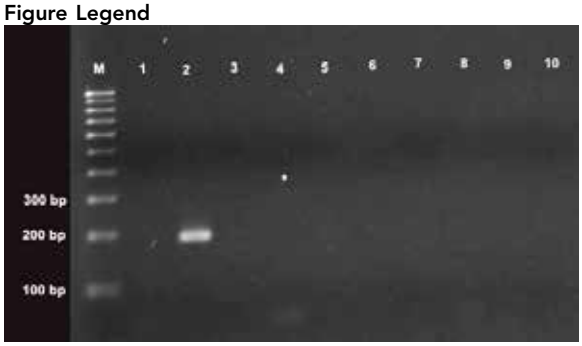

Fig. 2: 2% gel Electrophoresis shown ctxB-F4 with Rv-Cla for detecting the ctxB of classical genotype. Showing lane M 100 bp molecular weight marker, lane 1-N16961 El Tor control strain showing negative PCR product, lane 2-569B Classical strain showing 191 bp PCR product,lane3- DL32521 Haitian control strain showing negative PCR product, lane 4 Negative control and lane 5-10 test strains showing negative PCR product.

Table Legend

Table1. Cholera suspected cases and Positive case from different parts of Haryana in 2012

Area	Suspected Cholera Cases	Positive (%)	Sero-type	Sources	Year
Bahadur-garh	3	1(33.33%)	O1, Ogawa	Stool swab	2012
Bawal	1	0 (0%)	-	Stool swab	2012
Bullabgarh	1	1 (100%)	O1, Ogawa	Stool swab	2012
Faridabad	9	5 (55.55%)	O1, Ogawa	Stool swab	2012
Gurgaon	2	1 (50%)	O1, Ogawa	Stool swab	2012
Kadarpur	1	0 (0%)	O1, Ogawa	Stool swab	2012
Maneser	2	1 (50%)	O1, Ogawa	Stool swab	2012
Mewat	6	3 (50%)	O1, Ogawa	Stool swab	2012
Palwal	1	0 (0%)	-	Stool swab	2012
Sonipat	5	0 (0%)	-	Stool swab	2012

Table Legend

Table 2. Antibiotic patterns of *V. cholerae* O1 in Haryana in 2012

Strains ID	AM	AZM	CTX	CRO	C	CIP	COT	GEN	IPM	NA	NX	OF	TE
31698	S	S	S	S	S	S	R	S	S	R	S	S	R
31853	S	S	S	S	S	S	R	S	S	R	S	S	R
31860	S	S	S	S	S	S	R	S	S	R	S	S	R
31948	S	S	S	S	S	S	R	S	S	R	S	S	R
32285	IM	S	IM	IM	S	S	R	S	S	R	S	S	S
32286	IM	S	S	S	S	S	R	S	S	R	S	S	S
32289	IM	S	S	IM	S	S	R	S	S	R	S	S	S
32355	R	S	S	IM	S	S	R	S	S	R	S	S	S
32375	IM	S	S	S	S	S	S	S	S	R	S	S	S
32394	IM	S	S	IM	S	S	R	S	S	R	S	S	R
32626	IM	S	R	S	S	S	R	S	S	R	S	S	S
33102	IM	S	S	S	S	S	R	S	S	R	S	S	S
ATCC25 922	S	S	S	S	S	S	S	S	S	S	S	S	S
N16961	S	S	S	S	S	S	S	S	S	S	S	S	S
569B	S	S	S	S	S	S	S	S	S	S	S	S	S

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