Antimicrobial susceptibility and molecular characterization of *Vibrio cholerae* from cholera outbreaks in Chennai

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Abstract The genotype and antibiotic resistance pattern of the toxigenic *Vibrio cholerae* strains associated with cholera outbreaks vary frequently. Fifty-one *V. cholerae* strains isolated from cholera outbreaks in Chennai (2002–2005) were screened for the presence of virulence and regulatory genes by multiplex polymerase chain reaction (PCR) assay. Genotyping of the isolates was done by VC1 primers derived from enterobacterial repetitive intergenic consensus (ERIC)-related sequence in *V. cholerae*. All the isolates possessed toxigenic genes, such as *ctxA*, *ctxB*, *tcpA*, *ace*, *ompU*, *toxR* and *zot*. Two different El Tor genotypes and one O139 genotype could be delineated by VC1-PCR. One of the El Tor genotypes was similar to the El Tor strains isolated from Bhind district and Delhi during 2004. Antibiotic susceptibility testing revealed greater variability among the isolates tested. All the isolates were found to be susceptible to norfloxacin, ciprofloxacin and tetracycline. Thirty-three per cent of the isolates were found to be resistant to more than 4 antibiotics and could be termed as multiple antibiotic resistant. Coexistence of O139 serogroup along with the El Tor biotype could be identified among the strains recovered during the period 2002–2004. The O139 isolates were

found to be more susceptible to the antibiotics tested when compared to the El Tor isolates.

Keywords Antibiotics · DNA fingerprinting · VC1-PCR · *Vibrio cholerae* · Cholera

Introduction

Cholera outbreaks that mostly ravage the developing countries are caused by toxigenic *Vibrio cholerae* that may belong to O1 or O139 serogroup. There has been a constant flux in the serogroup and antibiotic resistance profile of *V. cholerae* isolates recovered from a particular region or regions separated spatially over a span of time. This is illustrated by the emergence of O139 serotype in 1992 and in 1996 and subsequent reclamation after a short period of prominence [1]. Although O139 serogroup is not associated with widespread cholera outbreaks during recent years, it could be seen occasionally in regions where cholera is endemic. Widespread resistance to antibiotics such as nalidixic acid, ciprofloxacin, co-trimoxazole, chloramphenicol, tetracycline, cephalixin and ampicillin has been reported in *V. cholerae* [2]. The drug resistance does not accrue in *V. cholerae* O1 and O139 as in the case of other pathogens like *Shigella* spp, but exhibit temporal and spatial fluctuation with periods of sensitivity, usually reflective of the antibiotics that are abused in any given region. This variation in the resistance pattern is due to the loss or acquisition of conjugative plasmids, conjugative integrons or SXT element which have been described as vehicles for the acquisition of resistance genes [3].

V. cholerae with varied genotypes has been isolated time and again from different cholera outbreaks [4, 5]. Variation

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in the genotype could be understood by different molecular typing methods, such as ribotyping, pulse field gel electrophoresis (PFGE), and PCR-based typing methods such as arbitrarily primed PCR (AP-PCR), repetitive extragenic palindrome PCR (REP-PCR), enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) and BOX-PCR. Different combinations of primers derived from repetitive DNA sequences have been successfully used in molecular typing of *V. cholerae* strains elsewhere. PCR DNA fingerprinting using the primers based on these repeat sequences are easy to perform and can very well aid in bringing out the relatedness or differences among the *V. cholerae* isolates [6].

Chennai is considered to be one of the endemic regions for cholera in India. We obtained *V. cholerae* strains isolated from diarrhoeal patients admitted to communicable diseases hospital at Chennai from 2002–2005. In this study, we examined these *V. cholerae* strains for their antimicrobial sensitivity and also used molecular tools to analyze their genotypes and compared them with the *V. cholerae* strains obtained from Madhya Pradesh and Delhi during the year 2004.

Materials and methods

Bacterial strains

Diarrhoeal outbreaks of smaller proportions occur in regions of North Chennai with the onset of northwest monsoon (August to November). Chennai experienced cholera outbreaks during January (90) – February (50) and June (70) – July (100) months of 2002, August (50) in 2003, May (250) and October (80) – November (250) months of 2004 and October (28) of 2005. (The number of positive cases recorded is given in brackets along with the respective months.) *V. cholerae* strains were isolated from stool samples of the diarrhoeal patients during these outbreaks. A total of 51 *V. cholerae* strains (41 were O1 serogroup and 10 were O139 serogroup) isolated from cholera patients hailing from different localities in Chennai during the period 2002–2005 were included in this study. Two El Tor strains each from Delhi and Bhind outbreaks and three reference strains one each of El Tor biotype, O1 Classical biotype and O139 serogroup obtained from National Institute of Cholera and Enteric diseases (NICED), Calcutta were also used for comparison.

Antibiotic sensitivity tests

Antimicrobial susceptibility of clinical isolates of *V. cholerae* was tested by the disc diffusion method on Muller Hinton

agar as per the guidelines of Clinical and Laboratory Standards Institute (CLSI) formerly National Committee for Clinical Laboratory Standards (NCCLS). All the clinical isolates grown up to their log phase were spread onto Muller Hinton Agar plate by sterile swabs. Sensitivity discs containing CLSI approved concentrations were obtained from Hi-Media, Bombay (India) and placed aseptically onto the plate and inhibition zones were measured. The antibiotic discs used in this study are given in Table 2. The isolates were scored as sensitive or resistant as per the CLSI guidelines [7].

Molecular characterization

The *V. cholerae* strains were reconfirmed by *ompW*-PCR [8] and further screened for presence or absence of the virulence and regulatory genes by using the multiplex PCR assay described elsewhere [9]. The *ompW* PCR was performed using the primers OWF- 5' CAC CAA GAA GGT GAC TTT ATT G 3'; OWR- 5' GAA CTT ATA ACC ACC CGC G 3' following the temperature profile of denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 sec annealing at 56°C for 1 min and extension at 72°C for 1 min. DNA fingerprinting by VC1 primers was carried out in a final volume of 20 µL using the primers and temperature ramping as described by Shankuan [10]. The reaction mixture contained 50 ng template DNA, 2 µL of 10X polymerase reaction buffer, 3 mM MgCl₂ (final conc); 200 µM each of (final conc) dATP, dCTP, dTTP and dGTP, 1.5 U Taq polymerase and 0.5 µM VC1 primer. For every PCR reaction, a negative control (no template DNA) and a positive control (template DNA giving amplified product) were maintained. The amplified product was run on a 1.2% agarose gel along with 1kb DNA ladder, at a constant voltage of 60V and visualized under UV light.

Results and discussion

All the *V. cholerae* strains used in the study were positive for *V. cholerae* specific *ompW* gene and produced an amplicon of 588 bp. The isolates were further positive for virulence and regulatory genes *ctxA*, *tcpA*, *ace*, *ompU*, *toxR* and *zot*. The *ompW* gene sequence is highly conserved among the *V. cholerae* belonging to different serogroups and is targeted for the species-specific identification of *V. cholerae*. The pathogenesis of cholera relies on a battery of virulence factors that are mostly located in CTX element and TCP Pathogenicity island. The *V. cholerae* isolates characterized in the present study possessed all the virulence associated genes which are required for pathogenesis.

PCR DNA fingerprinting using primers derived from repetitive elements (rep-PCR) has been described as a useful

tool in the assessment of the clonal relationship among the bacterial isolates as well as in the epidemiological analysis and examination of the genotypic diversity of *V. cholerae* strains [9]. Moreover, the rep-PCR methods have been reported to be more discriminatory and have the potential to substitute the cumbersome ribotyping [6]. The VC1 primer derived from the ERIC related sequence in *V. cholerae* was used in rep-PCR fingerprinting. The VC1-PCR of genomic DNA from the *V. cholerae* isolates resulted in DNA profiles comprising of multiple DNA fragments ranging from 0.25 kb to 2.5 kb (Fig. 1). All the 3 reference strains viz. O139, O1 Classical, O1 El Tor showed different patterns with this method. The El Tor reference strain and other El Tor strains recovered from Chennai used in the study showed a prominent band of 1400 bp, which was absent in O1 Classical and O139 strains. This prominent band could serve as a specific marker to identify toxigenic El Tor strains. All the El Tor and the O139 strains showed similar profile except for the absence of the 1400 bp band in O139 (Fig. 1). Two bands of approximately 375 bp and 380 bp could be seen in 16 El Tor strains, which differentiated them from the rest. These

two bands were not present in 4 representative El Tor strains of 2004 cholera outbreaks from Delhi and Bhind district of Madhya Pradesh. The 51 strains studied during the four-year period could be differentiated into two El Tor and one O139 genotype. VC1-PCR has the potential to differentiate the serogroups and biotypes of *V. cholerae*.

The antibiotic resistance profile of all the 51 *V. cholerae* strains against 17 different antimicrobial agents is shown in Table 1. Resistance or sensitivity to the antibiotics was determined as per the guidelines of CLSI. All the strains were found to be sensitive to ciprofloxacin, norfloxacin and tetracycline. Although ciprofloxacin resistance has been reported for the El Tor strains elsewhere [5, 11] the El Tor as well as the O139 strains recovered from the Chennai region during the year 2002–2004 were ciprofloxacin sensitive. Gentamycin and doxycycline were effective against majority of the *V. cholerae* strains except for the isolates C98 and C105, respectively. Relatively lesser resistance was seen in the case of antibiotics such as amikacin (6%), cephalixin (6%), while the strains analyzed in the study were most often resistant to the antibiotics nalidixic acid (96%),

Fig. 1 *V. cholerae* genotypes identified using VC1 primers. Lanes: 1. C7, 2. D4 (Delhi isolate), 3. C45, 4. C62, 5. C65, 6. C73, 7. C76, 8. C23 (O139 serogroup), 9. C86, 10. C91, 11. 102, Lane M: 1kb ladd

Table 1 Antibigram of the isolates used in the study

S. No.	Isolates	Antibiogram	S.No	Isolates	Antibiogram
1	C1	A, Cac, Co, E, Na, Tr	27	C72	Co, Na, Tr
2*	C2	Na	28	C73	Co, E, Na, Tr
3	C3	A, E, Na, Tr	29	C74	A, C, Cac, Co, E, Na, Pb, Tr
4*	C4	E, K, Na	30	C76	A, Am, Co, E, Na, Tr
5	C7	Co, E, Na, Tr	31	C77	A, Cac, Co, Na, Pb, Tr
6	C9	Co, E, K, Na, Tr	32	C80	Co, Na, Tr
7	C10	Co, E, Na, Tr	33	C81	A, Am, Cac, Co, Cp, E, Na, Tr
8	C11	Co, E, K, Na, Tr	34	C82*	-
9	C12	Co, E, Na, Tr	35	C84	A, Am, C, Co, Cp, E, Na, Tr
10*	C15	E, Na	36	C85	A, Co, E, Na, Pb, Tr
11*	C16	E, K, Na, Tr	37	C86	Co, E, Na, Tr
12	C17	Cac, E, K, Na, Tr	38	C91	Co, Na, Tr
13	C18	E, K,,Na	39	C93	Co, Na, Tr
14*	C23	E, Na	40	C95	Co, Na, Tr
15*	C28	Cac, E, K, Na	41	C97	Co, E, Na, Tr
16	C42	Co, Cp, E, Na, Tr	42	C98	A, Ak, Co, E, G, K, Na, Pb, Tr
17	C45	Co, Na Tr	43	C102	A, Co, Pb, Tr
18	C47	Co, Na, Tr	44	C105	A, Ak, Am, Co, Do, E, Na, Tr
19	C49	Ak, E, Na	45	C106	Co, E, K, Na
20*	C50	Na	46	C108	Co, E, Na, Tr
21*	C52	A, Cac, E, Na	47	C110	Co, E, Na, Tr
22	C62	Co, Na, Tr	48	C115	Co, Na, Tr
23	C65	Am, C, Co, E, Na, Tr	49	C116	A, Co, E, Na, Tr
24	C69	A, C, Co, E, Na, Tr	50	C117	Co, Na, Tr
25	C70	Co, Na, Tr	51	C111	Co, E, K, Na, Pb, Tr
26	C71	Co, E, Na, Tr			

*O139 isolates

trimethoprim (78%), co-trimoxazole (75%) and erythromycin (73%) (Table 2). Lesser percentage of resistance was noticed with respect to other antibiotics, which ranged from 8% to 27% (Table 2). Thirty-three per cent strains were found to be multiple antibiotic resistant (MAR: resistance to 4 or more antibiotics), which was found to vary among the individual isolates. Only one O139 *V. cholerae* isolate, C82 was found to be susceptible to all the antibiotics tested. On the other hand *V. cholerae* strain C98 exhibited the highest resistance (resistant to about 9 of the 17 antibiotics tested). Among the *V. cholerae* strains tested, O139 serogroup strains were found to show greater susceptibility to the antibiotics when compared to the O1 strains. The O139 strains from earlier outbreaks from 1999–2000 at six different places in India showed similar antibiogram among themselves [12], but the O139 strains from Chennai exhibited difference in susceptibility for the antibiotics tested. Wide variation in the antimicrobial susceptibility could be seen in

the case of the strains isolated during a particular year itself. Antimicrobial testing studies carried out onto the *V. cholerae* strains recovered from Delhi (21 strains) and Bhind (12 strains) during the same period showed a uniform pattern of susceptibility to the antibiotics amikacin, amoxycillin, ampicillin, chloramphenicol, clavulanic acid, ciprofloxacin, cephalixin, doxycycline, erythromycin, gentamycin, kanamycin, norfloxacin, tetracycline and resistance to nalidixic acid, polymyxin-B, trimethoprim and co-trimoxazole (data not shown). The 2 representative *V. cholerae* strains from Bhind and Delhi had similar antimicrobial sensitivity profile.

Though there are many reports about the involvement of a varied serogroups of *V. cholerae* in the diarrhoeal illness, only O1 and O139 serogroups have been widely isolated from epidemic like situations. The O139 serogroup strains which are routinely encountered in cholera cases that occur sporadically could be identified during the period

Table 2 Percentage of isolates resistant to different antibiotics

S. No.	Antibiotics	% isolates resistant		
		O1 El Tor	O139	Combined
1	Ampicillin (A)	32	10	27
2	Amikacin (Ak)	5	10	6
3	Amoxicillin (Am)	12	-	10
4	Chloromphenicol (C)	10	-	8
5	Clavulanic acid (Cac)	12	20	13
6	Ciprofloxacin (Cf)	-	-	-
7	Co-trimoxazole (Co)	92	-	75
8	Cephalexin (Cp)	7	-	6
9	Doxycycline (Do)	2	-	2
10	Erythromycin (E)	68	70	73
11	Gentamycin (G)	2	-	2
12	Kanamycin (K)	17	30	20
13	Nalidixic acid (Na)	97	90	96
14	Norfloxacin (Nx)	-	-	-
15	Polymyxin-B (Pb)	15	-	12
16	Tetracycline (T)	-	-	-
17	Trimethoprim (Tr)	95	10	78

2002–2004 among the *V. cholerae* strains isolated from the cholera patients in Chennai region. The two El Tor and one O139 VC1 genotypes identified among the *V. cholerae* strains isolated from Chennai had variable antibiograms. The variation in the antibiotic resistance profile may be due to the loss or acquisition of genetic determinants for drug resistance, which has been seen in earlier reports also, where isolates with different antibiograms found to show similar ribotypes [5]. Although rehydration therapy is the main stay of therapy for cholera, antimicrobials are important adjunct to therapy. Use of appropriate antimicrobials would markedly decrease overall stool volume and the length of illness. The presence of *V. cholerae* strains with varied antimicrobial sensitivity makes it difficult for the clinicians to adopt a common antimicrobial therapy for the patients admitted with cholera even during a particular year. Hence, the antimicrobial susceptibility tests needs to be carried out frequently along with *V. cholerae* identification when a patient presents with a severe watery diarrhea. Appropriate choice for empiric antimicrobial therapy still includes norfloxacin, ciprofloxacin, tetracycline and doxycycline, as the *V. cholerae* strains have not developed resistance to these antibiotics in this region.

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