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ORIGINAL PAPER

Molecular characterization of *Vibrio cholerae* outbreak strains with altered El Tor biotype from southern India

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Abstract Forty-four *Vibrio cholerae* isolates collected over a 7-month period in Chennai, India in 2004 were characterized for gene traits, antimicrobial susceptibility and genomic fingerprints. All 44 isolates were identified as O1 El Tor Ogawa, positive for various toxigenic and pathogenic genes viz. *ace*, *ctxB*, *hlyA*, *ompU*, *ompW*, *rfbO1*, *rtx*, *tcpA*, *toxR* and *zot*. Nucleotide sequencing revealed the presence of cholera toxin B of classical biotype in all the El Tor isolates, suggesting infection of isolates by classical CTXΦ. Antibiogram analysis showed a broad-spectrum antibiotic resistance that was also confirmed by the presence of resistant genes in the genomes. All isolates contained a class 1 integron and an SXT constin. However, isolates were sensitive to chloramphenicol and tested negative for the chloramphenicol resistant gene suggesting a deletion in SXT constin. Fingerprinting analysis of isolates by ERIC- and Box PCR revealed similar DNA patterns indicating the clonal dissemination of a single predominant *V. cholerae* O1 strain throughout the 2004 outbreak in Chennai.

Keywords *Vibrio cholerae* · Molecular characterization · Antibiogram · Toxin gene · Evolution

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Introduction

The disease cholera, caused by the bacteria *Vibrio cholerae,* is a continuous public health problem in countries with poor socio-economic conditions. Almost every developing country is facing either a cholera outbreak or the threat of an epidemic. Cholera has the potential to appear in explosive outbreak, epidemic or even pandemics. The world has already faced seven cholera pandemics in the past two centuries (Kaper et al. 1995). The Indian sub-continent has been an epicenter for cholera. Chennai, a metropolitan city in Southern India, is plagued by reoccurring cholera outbreaks in suburbs due to contamination of drinking water supplies. *V. cholerae* O139 strain, an epidemic strain that was unknown prior to 1991, emerged from this region and spread to the Southeast Asian countries.

The two biotypes of O1 serotype, El Tor and classical are believed to have evolved from separate lineages (Kaper et al. 1995). Between the two biotypes, El Tor strains have better adaptability to survive in the environment and in the human host as they colonize better the intestinal epithelium (Finkelstein 2006). Strains of classical biotype are suggested to be more toxigenic than El Tor strains (Huq et al. 1993). Recent studies have shown the spread of El Tor strains harbouring classical cholera toxin gene in US Gulf Coast and several countries in Asia and Africa (Nair et al. 2006; Goel et al. 2008).

In this study, we report molecular characterization of 44 *V. cholerae* O1 isolates from patients during a cholera outbreak in Chennai from April to November 2004. Strains were characterized for antimicrobial susceptibility, the presence of various toxigenic and pathogenic genes, nucleotide sequences of *ctxB* gene, and genomic fingerprints. This research contributes to our understanding of *V. cholerae* disease pattern and the evolution of pathogenicity.

Materials and methods

Bacterial cultures

The *V. cholerae* strains were isolated from stool samples of randomly selected patients during an outbreak in Chennai, India, between April and November 2004. Samples were collected using sterile rectal swabs from the patients admitted at Communicable Disease Hospital, Chennai as described earlier (Pourshafie et al. 2007). Forty-four isolates were randomly selected spanning the 7-month sampling period. *V. cholerae* was identified and confirmed by using standard biochemical methods (Tamrakar et al. 2006). Serological identification of the isolates was done by slide agglutination using commercially available polyvalent antiserum against *V. cholerae* O1 and O139 (Difco, USA). Biotype of isolates was confirmed by polymyxin B sensitivity and VP test. *V. cholerae* O1 (ATCC 11623) and *V. cholerae* O139 (ATCC 51394) were used as reference strains.

Biochemical characterization

All bacterial isolates were screened for oxidase reaction followed by other standard tests for presumptive identification of *V. cholerae* (Tamrakar et al. 2006). Serological identification of the isolates was done by slide agglutination using commercially available polyvalent antiserum against *V. cholerae* O1 and O139 (Difco, USA).

Antibiotic susceptibility

The antimicrobial susceptibility of the *V. cholerae* isolates was determined by the disc diffusion method on Mueller–Hinton agar as described by the Clinical Laboratory Standards Institute (CLSI 2007). The antibiotics discs were used at the following concentrations: ampicillin (10 μg), chloramphenicol (30 μg), ceftazidime (10 μg), ciprofloxacin (5 μg), co-trimoxazole (25 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), nitrofurantoin (300 μg), norfloxacin (10 μg), polymyxin-B (50 μg), spectinomycin (100 μg), streptomycin (10 μg), sulphamethizole (300 μg), sulphamethoxazole (100 μg) tetracycline (30 μg) and trimethoprim (5 μg). The antibiotic discs used in this study were purchased from HiMedia, India.

Detection of gene traits

Genomic DNA was extracted from each of the isolate using genomic DNA purification kit (MBI Fermentas, Vilnius, Lithuania) and screened for the presence of diverse gene traits by two sets of multiplex PCR as described elsewhere (Kumar et al. 2009). The first multiplex PCR revealed the presence of *ompW*, *ctxB*, *rfbO1*, *tcp* and *zot* genes. The second set of multiplex PCR detected *rtxC*, *ace*, *hlyA*, *ompU* and *toxR* gens. Primer sequences used for amplification of different genes are listed in Table 1. The specificity of PCR was confirmed by using reference bacterial strains. PCR amplification was carried out in a 25 μl reaction mixture using a Palm Cycler (Corbett Life Sciences, Australia). The reaction mixture contained 1× reaction buffer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 1 U of *Taq* polymerase (Fermentas), varying concentration of primers (see Table 1) specific for each target gene, 100 ng of template DNA and milli-Q water. The thermal cycling conditions for both the multiplex PCR were: pre-incubation at 94°C for 2 min, 30 cycles of 1 min at 94°C for denaturation, 1 min at 59°C for annealing and 2 min at 72°C for extension followed by incubation at 72°C for 10 min for final extension.

PCR for class 1 integron and SXT constin genes

Class 1 integron was detected by PCR using primers *qacEΔ1-F* and *sul1-B*, directed at the 3′-CS of class 1 integrons. To investigate the presence of SXT constin, a conjugative self transmissible plasmid, primers *int1-F* and *int1-B* specific for SXT integrase were used (Hochhut et al. 2001). The primer sequences and PCR conditions used for class 1 integrons and SXT constin were identical to those described previously (Hochhut et al. 2001). To verify the presence of antibiotic resistant gene cassettes among integrons, primers *in-F* and *aadA-B* were used to detect the presence of *aadA2* gene cassette encoding resistance to streptomycin and spectinomycin. Primers *in-F* and *blaP1-B* were used to detect the presence of *β*-lactam gene cassette in integrons (Dalsgaard et al. 2000). Isolates positive for SXT integrase gene were examined for the presence of the SXT-associated resistance genes, *floR* and *strA* (encoding for chloramphenicol and streptomycin resistance, respectively) by PCR as previously described (Hochhut et al. 2001).

Sequencing of cholera toxin (*ctxB*) gene

Cholera toxin B (*ctxB*) gene was amplified from the isolates using the *ctxF* and *ctxR* primers as described earlier (Olsvik et al. 1993). Sequencing was carried out using the same PCR primers on a 96 capillary model 3730xl system using the Big Dye Terminator kit from Applied Biosystems (Applied Biosystems, Foster City, CA, USA). The sequences were edited with SeqED program (Applied Biosystems), and were aligned with reference strains using ClustalW program. The sequences of *ctxB* gene for reference strains were retrieved from the public domain (GenBank).

Table 1 PCR primers used in this study

Primer	Nucleotide sequence	Amplicon size (bp)	Primer used in 25 µl RXN (pmol)	Annealing temperature (°C)	References
ompW (F)	GGTAGCCTTGGTGATATTGGTG	208	12	59	This study
ompW (R)	TAGCAGCAAGTCCCCATGAGT		12		
ctxB (F)	GCCGGGTTGTGGGAATGCTCCAAG	536	9	59	Goel et al. (2007)
ctxB (R)	CATGCGATTGCCGCAATTAGTATGGC		9		
rfbO1 (F)	TCTATGTGCTGCGATTGGTG	638	10	59	Goel et al. (2007)
rfbO1 (R)	CCCCGAAAACCTAATGTGAG		10		
tcp-F	CGTTGGCGGTCAGTCTTG	805	10	59	Goel et al. (2007)
tcp-R	CGGGCTTTCTTCTTGTTCG		10		
zot (F)	TCGCTTAACGATGGCGCGTTTT	947	8	59	Singh et al. (2001)
zot (R)	AACCCCGTTTCACTTCTACCCA		8		
rtxC-F	CGACGAAGATCATTGACGAC	265	10	59	Chow et al. (2001)
rtxC-R	CATCGTCGTTATGTGGTTGC		10		
ace-F	TAAGGATGTGCTTATGATGGACACCC	309	10	59	Kumar et al. (2009)
ace-R	CGTGATGAATAAAGATACTCATAGG		10		
hlyA-F	GAGCCGGCATTCATCTGAAT	480	10	59	Kumar et al. (2009)
hlyA-R	CTCAGCGGGCTAATACGGTTTA		10		
toxr-F	CCTTCGATCCCCTAAGCAATAC	779	10	59	Kumar et al. (2009)
toxr-R:	AGGGTTAGCAACGATGCGTAAG		10		
ompU-F:	ACGCTGACGGAATCAACCAAAG	869	10	59	Kumar et al. (2009)
ompU-R	GCGGAAGTTTGGCTTGAAGTAG		10		
ctx-F	GATACACATAATAGAATTAAGGATG	461	10	59	Olsvik et al. (1993)
ctx-R	GGTTGCTTCTCA TCATCGAACCAC		10		
ERIC-F	ATGTAAGCTCCTGGGGATTCAC	Variable	10	52	Rivera et al. (1995)
ERIC-R	AAGTAAGTGACTGGGTGAGCG				
BOX A1R	CTACGGCAAGGCGACGCTGACG	Variable	10	53	Versalovic et al. (1994)
qacEΔ1-F	ATCGCAATAGTTGGCGAAGT	800	10	58	Dalsgaard et al. (2000)
Sul1-B	GCAAGGCGGAAACCCGCC		10		
int1-F	GCTGGATAGGTTAAGGGCGG	592	10	55	Hochhut et al. (2001)
int1-R	CTCTATGGGCACTGTCCACATTG		10		
in-F	GGCATCCAAGCAGCAAGC		10		Collis and Hall (1992)
*aadA-R**	ATTGCCCAGTCGGCAGCG	650	10	55	Kazama et al. (1995)
*blaP1-R**	CTGGTTCATTTCAGATAGCG	874	10	52	Kazama et al. (1995)
strA-F	TTGATGTGGTGTCCCGCAATGC	383	10	57	Hochhut et al. (2001)
strA-R	CCAATCGCAGATAGAAGGCAA		10		
floR-F	TTATCTCCCTGTCGTTCCAGCG	526	10	57	Iwanaga et al. (2004)
floR-R	CCTATGAGCACACGGGGAGC		10		

* Paired with *in-F*

Nucleotide sequence accession number

The nucleotide sequences obtained for the *ctxB* gene of strains VCC1588, VCC1969, VCC2411, VCC2491, VCC4372, VCC5910, VCC5963 and VCC5988 have been deposited in GenBank under accession number EU428013, EU428014, EU428015, EU428016, EU428017, EU428018, EU428019 and EU428020, respectively.

Genomic DNA fingerprinting analyses

Enterobacterial repetitive intergenic consensus (ERIC) sequence PCR was performed as described earlier with little modifications by using two oligonucleotides ERIC-F and ERIC-R (Rivera et al. 1995). The thermal cycler was programmed for 35 cycles of 1 min at 94°C, 1 min at 52°C, 10 min at 68°C followed by 20 min incubation at 70°C.

BOX PCR was performed by using a single nucleotide primer BOX A1R (Versalovic et al. 1994). PCR program consisted of: initial denaturation (95°C for 7 min), 30 cycles of 94°C for 1 min, 53°C for 1 min, 65°C for 8 min, and a final extension of 65°C for 16 min.

Results and discussion

Strain identification and detection of gene traits

A total of 44 bacteria isolated from the patients were biochemically identified as *V. cholerae* and serologically confirmed as O1 Ogawa. Multiplex PCR analysis revealed that all strains tested positive for *ace*, *ctxB*, *hlyA*, *ompU*, *ompW*, *rfbO1*, *rtx*, *tcpA*, *toxR* and *zot genes* (Fig. 1). The outer membrane gene (*ompW*) is species specific for *V. cholerae* and its presence confirms the biochemical identification of *V. cholerae*. Somatic O-antigen biosynthesis gene was positive for O1 type (*rfbO1)*, providing molecular evidence for O1 serogroup. Collectively, the results indicated that 2004 Chennai outbreak was caused by a toxigenic *V. cholerae* O1 Ogawa.

To identify the biotype of the isolates, VP test, polymyxin B resistant test and *rtx*C PCR were performed. Results showed that all isolates were VP negative, resistant to polymyxin B and PCR positive for repeat in toxin (RTX) gene C (*rtx*C). Reference *V. cholerae* O1 classical (ATCC 11623) strain was PCR negative for RTX gene. Both negative VP test and polymyxin B resistance suggested that Chennai isolates belonged to El Tor biotype. The RTX represents a family of important virulence factors that have disseminated widely among gram-negative bacteria (Coote 1992). The RTX gene cluster in *V. cholerae* encodes the presumptive cytotoxin (*rtxA*), an acyltransferase (*rtxC*), and an associated ATP-binding cassette transporter system (Lin et al. 1999). PCR assays developed for *rtxA* or *rtxC* in *V. cholerae* can differentiate El Tor biotype from classical biotype among O1 serogroup (Chow et al. 2001). The positive amplification of *rtx*C gene among the isolates further confirmed El Tor biotype.

In addition to *ctxB* and *rtxC* genes, the isolates were also positive for haemolysin (*hlyA*), zonula occludens (*zot*), accessory cholera toxin (*ace*), toxin coregulated pilus (*tcp*), outer membrane protein (*ompU*) and *toxR* genes. Cholera pathogenesis is a complex process and involves synergetic action of several genes. Although cholera toxin (CT) is supposed to be the most important epidemic marker among various toxins produced by *V. cholerae* (Kaper et al. 1995), CT is encoded by a mobile element, the genome of a filamentous CTX bacteriophage. The acquisition of CTXϕ relies on the presence of toxin co-regulated pilus (encoded by *tcp* genes) on cell surface, which acts as a receptor for CTXϕ infection (Waldor and Mekalanos 1996). Zonula occludens toxin (*zot*) and accessory cholera toxin (*ace*) genes are, in fact, genes encoding for CTXϕ structural proteins (Waldor and Mekalanos 1996). The simultaneous detection of the suite of ancillary toxin genes with *ctxB* gene confirmed the presence of CTX prophage in Chennai isolates.

Alteration of *ctxB* gene among El Tor strains

Figure 2 shows the amino acid sequences deduced from the nucleic acid sequences of *ctxB* gene. Interestingly, the CTX B sequences from the Chennai outbreak strains aligned with O1 classical reference strains. The deduced amino acid sequences differed from that of the reference El Tor strain by a histidine at position 39 and a threonine at position 68. Thus, the results indicated that Chennai El Tor strains contained an altered cholera toxin gene. It is likely caused by infection by classical CTXΦ. More accurately, Chennai outbreak was caused by a "hybrid" El Tor strain with classical phage. Similar results have been reported earlier from other countries in Asia and Africa (Nair et al. 2006; Safa et al. 2008) and more recently in eastern India (Kumar et al. 2009). The discovery of El Tor strains with classical *ctxB* gene in Chennai outbreak implied a widespread of El Tor strains with the "newly acquired" classical toxin gene in the Indian subcontinent.

The seventh and current pandemic of cholera is caused by the El Tor biotype. The classical biotype is believed to be extinct (Nair et al. 2002; Safa et al. 2008). The classical and El Tor biotypes of *V. cholerae* O1 are closely related in their O-antigen biosynthetic genes. However, the genomic structure of the CTXΦ, in which the cholera toxin genes are contained, differs between the classical and El Tor biotypes (Waldor and Mekalanos 1996; Ansaruzzaman et al. 2004). The results from India and Bangladesh showed that classical CT producing El Tor strains are now replacing the seventh pandemic El Tor strains (Nair et al. 2002, 2006; Kumar et al. 2009). This is taken as an evolutionary optimization of the El Tor biotype, which could represent a new and more significant emerging form of the El Tor biotype of *V. cholerae*.

Antibiotic resistant and mobile elements

Antibiotic susceptibility test revealed that all 44 isolates were sensitive to ampicillin, ceftazidime, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, norfloxacin and tetracycline. However, the isolates exhibited high rate of resistance towards co-trimoxazole, nalidixic acid, nitrofurantoin, spectinomycin, streptomycin, sulphamethizole, sulphamethoxazole and trimethoprim. All the isolates were resistant to polymyxin-B, a typical feature of El Tor

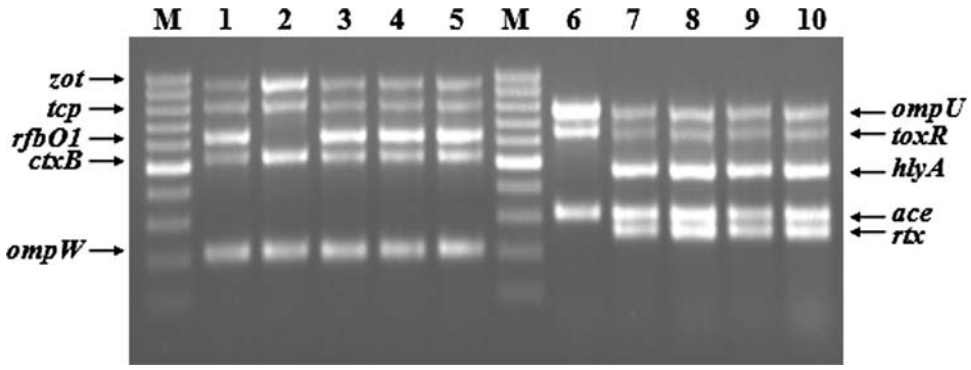

Fig. 1 Multiplex PCR 1 (*Lanes 1–5*) and multiplex PCR 2 (*Lanes 6–10*) for analysis of various genes. *Lanes 1 and 6*: *V. cholerae* O1 (ATCC 11623); *lanes 2 and 7*: *V. cholerae* O139 (ATCC 51394); *lanes 3 and 8*: *V. cholerae* VCC1969; *lanes 4 and 9*: *V. cholerae* VCC2411; *lanes 5 and 10*: *V. cholerae* VCC5988; *lane M*: 100 bp ladder

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                                    1         10        20        30        40        50        60
                                    |         |         |         |         |         |         |
N16961 ElTor Reference (NC-002505)  MIKLKFGVFFTVLLSSAYAHGTPQNITDLCAEYHNTQIYTLNDKIFSYTESLAGKREMAI
569B Classical Reference (U25679)   ....................................H.....................
        VCC1588 ElTor (EU428013)    ....................................H.....................
        VCC1969 ElTor (EU428014)    ....................................H.....................
        VCC2411 ElTor (EU428015)    ....................................H.....................
        VCC2491 ElTor (EU428016)    ....................................H.....................
        VCC4372 ElTor (EU428017)    ....................................H.....................
        VCC5910 ElTor (EU428018)    ....................................H.....................
        VCC5963 ElTor (EU428019)    ....................................H.....................
        VCC5988 ElTor (EU428020)    ....................................H.....................

                                   61        70        80        90       100       110       120
                                    |         |         |         |         |         |         |
N16961 ElTor Reference (NC-002505)  ITFKNGAIFQVEVPGSQHIDSQKKAIERMKDTLRIAYLTEAKVEKLCVWNNKTPHAIAAI
569B Classical Reference (U25679)   .......T.................................................
        VCC1588 ElTor (EU428013)    .......T.................................................
        VCC1969 ElTor (EU428014)    .......T.................................................
        VCC2411 ElTor (EU428015)    .......T.................................................
        VCC2491 ElTor (EU428016)    .......T.................................................
        VCC4372 ElTor (EU428017)    .......T.................................................
        VCC5910 ElTor (EU428018)    .......T.................................................
        VCC5963 ElTor (EU428019)    .......T.................................................
        VCC5988 ElTor (EU428020)    .......T.................................................
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Fig. 2 Amino acid sequence alignment of CTX-B subunit of *V. cholerae* O1 El Tor strains from Chennai outbreak with reference El Tor and classical strains. Identical amino acid residues are indicated by *dots*. Amino acid sequences of *V. cholerae* CTX-B reference strains used in alignment were retrieved from GenBank

biotype strains. Multiple antibiotic resistance among *V. cholerae* has emerged as a major problem worldwide (Faruque et al. 2007). In India, there is a progressive increasing trend of antibiotic resistance towards common fluoroquinolone, i.e., ciprofloxacin and norfloxacin since 1996 (Garg et al. 2001; Krishna et al. 2006). In this study, however, strains are sensitive to ciprofloxacin and norfloxacin but resistant to nalidixic acid, a non-fluorinated quinolone. In addition, isolates were sensitive to tetracycline, which is different from reports from other regions of India showing a re-emergence of tetracycling resistant strains (Jesudason 2006). Multi-drug resistance among *V. cholerae* strains limits the therapeutic potential of these drugs. Some antibiotics are already deemed unsuitable for certain group of population, i.e., tetracycline is not recommended for use in children and quinolone are not recommended in pregnant women and children (Sabeena et al. 2001). Multi-drug resistance presents additional challenges to disease management.

PCR results showed positive amplification of class 1 integron from Chennai isolates (Fig. 3). Integrons are an important mechanism for the acquisition of antibiotic resistance genes in many bacteria (Hall and Collis 1995). These elements are not autonomously mobile but are able

to capture, integrate and express resistance gene cassettes in their variable region. In this study, isolates were found to have integrons on the basis of PCR with 3′CS conserved sequence. PCR results also confirmed the presence of *aadA2* gene cassettes within the integron. The *aadA2* gene cassettes encode aminoglycoside adenylyltransferases inactivating streptomycin and spectinomycin (Recchia and Hall 1995). These gene cassettes are among the most prevalent gene cassettes in class 1 and class 2 integrons. All isolates were negative for *blaP1,* the *β*-lactam gene cassette, corresponding to the phenotypic sensitivity of Chennai strains to ampicillin and ceftazidime.

Recent studies suggest SXT constin as an important element for horizontal dissemination of antibiotic resistant genes in bacteria (Beaber and Waldor 2004). In SXT constins, the antibiotic resistance genes are clustered within a composite transposon-like structure found near the 5′ end of SXT. These genes confer resistance to chloramphenicol, sulphamethoxazole, streptomycin and trimethoprim (Beaber et al. 2002). In this study, all strains were found PCR positive for SXT integrase gene suggesting the presence of STX constin (Fig. 3). They were resistant to sulphamethoxazole, streptomycin and trimethoprim but were sensitive to chloramphenicol. The presence of *strA* gene within SXT was confirmed by PCR, which mediates resistance to streptomycin by phosphotransferase enzymes (aminoglycoside-3′-phosphotransferase and aminoglycoside-6′-phosphotransferase (Shaw et al. 1993). However, all isolates were PCR negative for *floR* gene, which is responsible for resistance towards chloramphenicol (Fig. 3). The deletion of *floR* gene within SXT contin suggests evolution of resistance characteristics through mobile element. SXT variants have been reported among clinical *V. cholerae* isolates in recent years (Faruque et al. 2003; Iwanaga et al. 2004). Early reports indicated the loss of resistance to sulphamethoxazole and trimethoprim, and showed varying resistance to streptomycin (Mukhopadhyay et al. 1998; Faruque et al. 2003). The new SXT variant found in Chennai isolates reconfirmed the importance of mobile element in the development of antibiotic resistance. We have demonstrated a direct correlation between the antibiotic resistant phenotype and the presence of drug resistance genes in Class 1 integron and SXT constins among Chennai isolates.

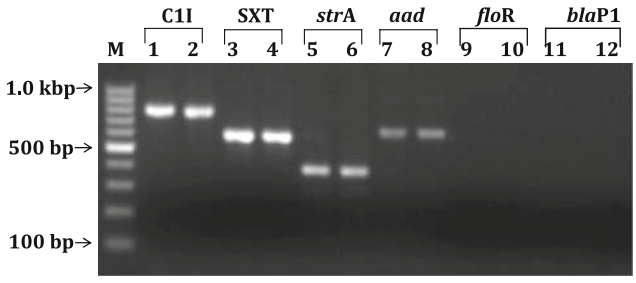

Fig. 3 PCR detection of class 1 integron, SXT constin and the associated antibiotic resistance genes. *Lanes 1, 3, 5, 7, 9 and 11*: Chennai isolate VCC1969; and *lanes 2, 4, 6, 8, 10 and 12*: Chennai isolate VCC5988. *Lane M*: 100 bp ladder

DNA fingerprinting analyses

All 44 *V. cholerae* isolates were characterized by ERIC- and BOX PCR to reveal the clonal relationship. ERIC PCR with genomic DNA of *V. cholerae* strains resulted in amplification of multiple fragments of DNA ranging between 0.25 and 1.8 kb. The BOX PCR of genomic DNA resulted in fragments varying between 0.65 and 6.0 kb. The fingerprinting analysis revealed identical DNA banding pattern among all 44 isolates, suggesting that a single clone of *V. cholerae* likely caused the Chennai outbreak over the 7-month period.

In summary, the study demonstrated that *V. cholerae* O1 El Tor biotype was responsible for the seven-month Chennai outbreak in 2004. This El Tor strain, however, contained classical cholera toxin gene, suggesting infection of isolates by classical CTXΦ. Mobile elements in the strain conferred multiple antibiotic resistances. All the isolates were derived from a single clone that initiated the outbreak in the area. This study calls attention for continuous monitoring of outbreak strains to understand the evolution of pathogen and disease pattern.

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