Retrospective genomic analysis of *Vibrio cholerae* O1 El Tor strains from different places in India reveals the presence of *ctxB-7* allele found in Haitian isolates

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SUMMARY

A total of 45 strains of *Vibrio cholerae* O1 isolated from 10 different places in India where they were associated with cases of cholera between the years 2007 and 2008 were examined by molecular methods. With the help of phenotypic and genotypic tests the strains were confirmed to be O1 El Tor biotype strains with classical *ctxB* gene. Polymerase chain reaction (PCR) analysis by double – mismatch amplification mutation assay PCR showed 16 of these strains carried the *ctxB-7* allele reported in Haitian strains. Sequencing of the *ctxB* gene in all the 45 strains revealed that in 16 strains the histidine at the 20th amino acid position had been replaced by asparagine and this single nucleotide polymorphism did not affect cholera toxin production as revealed by beads enzyme-linked immunosorbent assay. This study shows that the new *ctxB* gene sequence was circulating in different places in India. Seven representatives of these 45 strains analysed by pulsed – field gel electrophoresis showed four distinct *Not I* digested profiles showing that multiple clones were causing cholera in 2007 and 2008.

Key words: *ctxB-7*, molecular typing, PFGE, Surveillance, *Vibrio cholerae*.

INTRODUCTION

Vibrio cholerae is the etiologic agent of cholera, the acute diarrheal illness that continues to be a public health threat worldwide [1]. The pathogen has been traditionally classified into two biotypes, the classical and El Tor, based on clear phenotypic and genotypic differences. It has caused seven pandemics. The fifth and sixth pandemics occurred due to infection by the classical biotype while the current seventh pandemic that started in 1961 is due to the El Tor biotype [2]. The El Tor *V. cholerae* has undergone rapid genetic modifications due to the acquisition of different virulence gene cassettes by bacteriophage infection [3] and genetic recombination [4]. The exchange of genetic material that takes place in either the aquatic environment [5] or in the human GI system [6] has consequently led to the diversification of the seventh pandemic prototype El Tor strains into its different kinds of variants [7]

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among which the altered El Tor strains [8] have spread to different regions of the world from the point of its origin. With the advent of the hybrid and variants, identification of biotype based solely on phenotypic tests became impossible [9]. As a result molecular typing techniques became the reliable method of classification. This study was designed to determine the biotype of the strains by molecular methods and examine the genotypic characteristics of strains of *V. cholerae* O1 isolated in India between the years 2007 and 2008 from 10 different places.

METHODS

Bacterial strains included in the study

A total of 45 strains of *V. cholerae* from Bihar, Orissa, Chennai, Delhi, Guwahati, Kolkata, Pune, Mumbai, Rohtak and Surat as shown in Table 1 along with their year of isolation, were isolated from stool samples of cholera patients during the years 2007 and 2008 and sent to Vibrio Phage Laboratory at the National Institute of Cholera and Enteric Diseases in nutrient agar stabs for confirmation, biotyping, serotyping and phage typing. These served as representative strains causing cholera in India during 2007 and 2008.

Culture and phenotypic identification

Culture of *V. cholerae* was done by standard method by inoculating a loopful of culture from nutrient stab into 2 ml of APW (Alkaline Peptone Water) containing 1% NaCl and incubated for 6 hours at 37 °C with shaking. A loopful of the inoculum was transferred onto TCBS plate and streaked and the plates were incubated overnight at 37 °C and examined for typical round yellow *V. cholerae* colonies.

Further identification was done using standard conventional biochemical methods [10]. The strains were serogrouped using polyvalent O1 antisera from Denka Seiken (Tokyo, Japan) and serotyped using monovalent Ogawa and Inaba antisera from Denka Seiken (Tokyo, Japan). Phenotyping for biotype identification was done using Voges–Proskauer test, Polymixin-B sulfate sensitivity test and phage typing using lytic phages Mukerjee classical phage IV and Mukerjee El Tor phage V according to standard diagnostic procedures [11].

Detection of virulence genes by polymerase chain reaction (PCR)

PCR analysis was used to detect the major genes associated with *V. cholerae* virulence using specific primers

Table 1. *Places from where 45 representative strains from the years 2007 and 2008 were included in this study*

Sl. no.	Place	Total no. of strains	Strains in 2007	Strains in 2008
1.	Orissa	6	5	1
2.	Guwahati	4	4	0
3.	Pune	6	4	2
4.	Delhi	4	1	3
5.	Mumbai	1	0	1
6.	Surat	3	0	3
7.	Rohtak	1	1	0
8.	Chennai	4	3	1
9.	Kolkata	14	8	6
10.	Bihar	2	0	2
	Total	45	26	19

Table 2. Consequently, virulence genes *rstR*, *tcpA*, *ctxA*, *tcpI*, *rtxA*, *rtxC*, *zot*, *toxR*, *VC1449*, *VC1450*, *VCAO417*, *VCAO316*, *VCAO728*, *VCAO729*, *VCAO730*, *nag*, *nan*, *rstC*, *intl*, *orfU*, *acfB*, *tlc* were amplified, and these served as markers for the detection of the presence of various virulence regions of the *V. cholerae* genome. For screening the presence of VSP-I, *VCO183* was amplified while for detecting the presence of VSP-II region, the ORFs *VCO511*, *VCO513*, *VCO514*, *VCO515* and *VCO516* were amplified. *V. cholerae* O1 El Tor strain, N16961 and the O1 classical strain O395 were used as standard controls strains.

For PCR template, 1 ml of overnight culture was taken and centrifuged at 6000 rpm (Biofuge, Heraeus, Germany) for 5 min. The cell pellet was collected and resuspended in 300 µl of sterile distilled water. The cell suspension was boiled for 10 min in a boiling water bath and immediately transferred to ice for 10 min followed by centrifugation at 12 000 rpm for 10 min. 2 µl of this supernatant was used as template for PCR reaction in 20 ul PCR mixture containing 1U of 10× Taq DNA Polymerase (Bangalore Genei). The same cycling conditions as described in previous studies were used for PCR reaction [12]. PCR products were run on 2% agarose gels and examined using a gel-documentation system from Bio-Rad.

Mismatch amplification mutation assay (MAMA) PCR and double-mismatch amplification mutation assay (D-MAMA) PCR

MAMA PCR was used to detect the presence of *ctxB* gene and the type of biotype specific gene for cholera toxin B subunit present in the 45 test strains by

Table 2. *Sequence of PCR primers used in this study*

ORFs	Primer sequence (5′–3′)	Product size (bp)	Reference
ctxB Fw-con	ACTATCTTCAGCATATGCACATGG	186	Morita *et al.* [13]
ctxB Rv-cla	CCTGGTACTTCTACTTGAAA**CG**		
ctxB Rv-elt	CCTGGTACTTCTACTTGAAA**CA**		
ctxA F	CTCAGACGGGATTTGTTAGGCACG	302	Keasler *et al.* (1993)
ctxA R	TCTATCTCTGTAGCCCCTATTACG		
tcpA^{cla} F	CACGATAAGAAAACCGGTCAAGAG	618	
tcpA^{cla} R	ACCAAATGCAACGCCGAATGGAG		
tcpA^{elt} F	GAAGAAGTTTGTAAAGAAGAACAC	472	
tcpA^{elt} R	GAAAGGACCTTCTTTCACGTTG		
toxR F	CCTTCTGATCCCCTAAGCAATAC	779	Singh *et al.* (2001)
toxR R	AGGGTTAGCAACGATGCGTAAG		
zot F	TCGCTTAACGATGGCGCGTTTT	947	Singh *et al.* (2001)
zot R	AACCCCGTTTCACTTCTACCCA		
rtxC F	CGACGAAGATCATTGACGAC	265	Chow *et al.* (2001)
rtxC R	CATCGTCGTTATGTGGTTGC		
rtxA F	CTGAATATGAGTGGTTGACTTACG	418	Chow *et al.* (2001)
rtxA R	GTGTATTGTTCGATATCCGCTACG		
rtxA1	GCGATTCTCAAAGAGATGC	1366	Lin *et al.* (1999)
rtxA2	CACTCATTCCGATAACCAC		
tlc3	GGGAATGTTGAGTTCTCAGTG	1548	Rubin *et al.* (1998)
tlc4	GTTGCGAAGTGGATTTTGTG		
intl4 : 3	CCTTCATTGGATCACTCG	597	O'Shea *et al.* [12]
intl4 : 4	GACGGAAAAAGATAGTGCC		
acfB1	GATGAAAGAACAGGAGAGA	1000	O'Shea *et al.* (2002)
acfB2	CAGCAACCACAGCAAAACC		
orfUF	CGTCACACCAGTTACTTTTCG	1072	O'Shea *et al.* (2002)
orfU R	AGAATGTACGCCATCGC		
rstC1	AACAGCTACGGGCTTATTC	238	Waldor *et al.* (1997)
rstC2	TGAGTTGCGGATTTAGGC		
rstC F	ATGAGTTTGAAACCATACACTTT	238	Waldor *et al.* (1997)
rstC R	TTACAGTGATGGACTCAGTCAAT		
rstA1	ACTCGATACAAACGCTTCTC	1009	O'Shea *et al.* (2002)
rstA2	AGAATCTGGAAGGTTGAGTG		
tcpI F	TAGCCTTAGTTCTCAGCAGGCA	862	Rivera *et al.* (2001)
tcpI R	GGCAATAGTGTCGAGCTCGTTA		
rstR₁(forward)	CTTCTCATCAGCAAAGCCTCCATC	500	Nusrin *et al.* [21]
rstR₂(forward)	GCACCATGATTTAAGATGCTC		
rstA3(reverse)	TCGAGTTGTAATTCATCAAGAGTG		
VSP-I			
VCO183F	CAGTAAGAGTGTAGCGTGCC	950	O'Shea *et al.* [12]
VCO183R	CCTGCACATCGAGATGC		
VSP-II			
VC0511F1	CTTGCTGCGTACTTAGCA	385	Nusrin *et al.* (2009)
VC0511R1	AGTAGCATCGCTCTCGTA		
VC0513F1	CTGAGGTGTTATATGTTTCG	781	Nusrin *et al.* (2009)
VC0513R1	TCAAATTTCCTGACAGTTCC		
VC0514F1	GTTTGGGAAGGGTACACA	1·651	Nusrin *et al.* (2009)
VC0514R1	GCTCTTCAGCCGCTGA		
VC0515F1	GGTGGTGCTGCATGGA	1·135	Nusrin *et al.* (2009)
VC0515R1	TCTAAAGCCTCACACCA		
VCO516F	GTTTTCTGCGTTGTTCGAG	950	O'Shea *et al.* [12]
VCO516R	TCCTGATGTCTCTCTTGCCG		

employing primers Fw-con and Rv-cla and Rv-elt [13]. The reaction conditions for MAMA PCR was followed according to standard technique described previously [13]. D-MAMA PCR was used to detect the presence of *ctxB-7* allele in the strains tested by employing the same primers and cycling conditions as recently described [14].

Sequencing of the *ctxB* gene

Genomic DNA was extracted and purified using the method of Sambrook *et al.* [15] and used for PCR amplification of the 460 bp *ctxB* gene using the primer set *ctxB*-F 5′-(GGTTGCTTCTCATCATCGAACC AC)-3′ and *ctxB*-R 5′-(GATACACATAATAGAA TTAAGGATG)-3′. PCR was carried out in 25 µl reaction mixture containing 2·5 µl of 10× PCR (Takara Shuzo, Otsu, Japan), 2 µl of dNTP mixture (concentration 2·5 mM each) (Takara), 1 µl of each primer (10 pmol/µl), 0·2 µl (1U) of rTaq DNA polymerase (Takara) and 40 ng of chromosomal DNA of *V. cholerae* as template.

The PCR product was purified using Qiaquick PCR purification kit from Qiagen, GmBH, Germany by loading into purification column (Qiaquick spin column, Qiagen). A 40 ng of the purified PCR product was used in the cycle sequencing reaction mixture comprising the sequencing premix obtained from Perkin Elmer, USA, that consisted of Big Dye Terminator 3.1 Sequencing Buffer (5×) and ready to use reaction mixture (2·5×) and Big Dye Terminators (Perkin Elmer, USA) with AmpliTaq FS polymerase. An automated thermal cycler (Perkin Elmer Thermocycler 2400, USA) was used to amplify the 460 bp gene using 25 cycles of denaturation at 94 °C for 10 s, annealing at 50 °C for 5 s and extension at 60 °C for 4 min [16]. The product was purified and dried and sequenced using the DNA Analyzer 3730 automated sequencer (Applied Biosystems, Hitachi). Sequence editing and analysis was done using SeqMan (DNASTAR Inc., USA) Sequences were compared with sequences of control strains O395 (classical) and N16961 (El Tor) with accession numbers NC_012582 and AE003852.1 respectively from GenBank, NCBI and multiple alignments were performed using ClustalW of MEGA 5.0 [17] with gap open penalty = 10 and gap extension penalty = 0·2. The phylogenetic tree was constructed using neighbour-joining method with the bootstrapping of 1000 replications and 70 000 random seeds.

Beads enzyme-linked immunosorbent assay (ELISA) for quantification of cholera toxin (CT) production

The amount of CT produced by the 45 *V. cholerae* strains was measured by Beads ELISA method, which uses polystyrene beads of 6·5 mm in diameter coated with anti-CT IgG as a solid phase. The coated bead was first incubated with the sample and then incubated with anti-CT IgG [F(ab)]-horseradish peroxidase conjugate. Peroxidase activity was determined colorimetrically with 3,3,5,5-tetramethylbenzidine as the substrate by measuring the absorbance value at 450 nm. For this assay *V. cholerae* strains were streaked onto 1% nutrient agar plates and left for overnight incubation at 37 °C. Overnight culture was transferred to AKI medium and incubated at 37 °C for 20 h without shaking. Assay of cholera toxin was carried out according to the method described by Oku *et al.* [18].

Pulsed-field gel electrophoresis

Seven of the *V. cholerae* strains were analysed by PFGE to detect polymorphisms and consequential changes in restriction sites across their genome. Bacterial strains were cultured and agarose-embedded DNA plugs were prepared and digested with the enzyme *NotI* [19]. *Salmonella* serotype Braenderup H9812 was used as the molecular standard [20]. Agarose plugs were loaded onto a 1% agarose gel (Bio-Rad) and electrophoresis was carried out by using a contour-clamped homogenous electrical field Mapper (CHEF-DR III) (Bio-Rad Laboratories, Richmond, CA, USA) PFGE System following the electrical parameters as described earlier [19]. The PFGE gel was visualized and profile images were captured with Gel Doc XR (Bio-Rad Laboratories, Hercules, CA, USA) scanned and saved in the TIFF format using the Quantity One program (Bio-Rad Laboratories, Hercules, CA, USA) for analysis. The PFGE profiles were analysed and compared using the BioNumerics version 4.1 software (Applied Maths, Sint-Martens-Latem, Belgium). Similarity was based on the dice-coefficient and clustering analysis was performed and dendogram constructed with BioNumerics using the unweighted pair groupmatching algorithm (UPGMA) with a band position tolerance of 1·5%. A profile that differed by at least one clear band was considered a distinct profile. 18–20 bands were considered for comparison. Patterns with less than a four-band difference were considered subtypes.
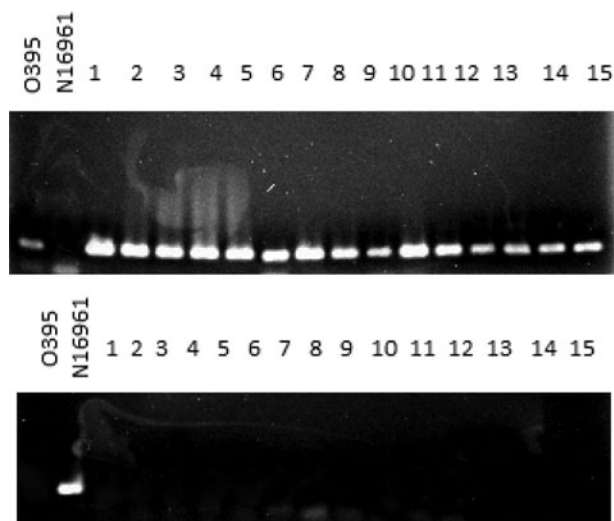
RESULTS

Phenotypic identification and detection of virulence genes

The 45 *V. cholerae* strains were confirmed to belong to the O1 serogroup and Ogawa was the dominant serotype. These strains showed phenotypic properties typical of the El Tor biotype.

PCR amplification for the detection of 28 major virulence genes (Table 3) showed that all of these strains had *ctxA* gene and El Tor specific *tcpA* gene proving that they were toxin producing strains of the El Tor biotype. The *rstR* gene amplified using biotype specific primers [21] successfully produced the 500 bp DNA fragment in all the strains with the *rstR* El Tor biotype specific primer pair while with *rstR* classical biotype specific primers there was no amplification of the *rstR* gene except in the classical standard control strain O395.

PCR for detection of CTXφ in the small chromosome in these strains that was done using the set of primers CIIF and CIIR [22] led to the amplification of 766 bp DNA fragment revealing the absence of a second copy of CTXφ in the small chromosome.

PCR confirmed the presence of *rstC, rtxA, rtxC* genes and also a set of five genes (*VCA0316, VCA0417, VCA0728, VCA0729, VCA0730*) present only in El Tor strains and absent in classical strains [23].

Successful PCR amplification of *tcpI, toxR, zot, ptlc, VC1449, VC1450, orfU, intl3, acf, nag, nan, rstA* genes using previously described primer sequences [12] confirmed the presence of all these ORFs and their corresponding virulence regions in the genome. Likewise, amplification of the *VC0183* gene confirmed the presence of VSP-I [12] while the successful amplification of *VC0513, VC0514, VC0515* and *VC0516* genes confirmed the presence of VSP-II region [12]. However, the absence of ORF *VC0511* in 19 of these 45 strains was revealed by the failure to amplify the expected 385 bp amplicon using primer sequence as previously described [12].

Sequencing of *ctxB* to determine the genotype of CT in these strains

MAMA PCR revealed the presence of *ctxB* gene of the classical biotype in all the strains (Fig. 1). However, D-MAMA PCR showed that 16 of these strains failed to produce DNA product using primers *ctxB*-F4/Rv-cla [14] while they successfully produced 191 bp product (Fig. 2) with primer pair ctxB-F3/

Table 3. *Result of virulence gene profiling done with the aid of PCR*

Virulence Regions (ORFs)	CTXφ				RS2		RS1φ		RTX				VPI-1				VPI-2		Chromosome2					TLC	VSP-I	VSP-II					Integron
Place, no. of strains	ctxA	ctxB	zot	orfU	rstR	rstA	rstC	rstC1C2	VC1449	VC1450	rtxA	rtxC	tcpA	tcpI	toxR	acfB	nagC	nanH	VCA0316	VCA0417	VCA0728	VCA0729	VCA0730	pTLC	VCO183	Vco511	Vco513	Vco514	Vco515	Vco516	Intl3:4
Orissa,5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+
Orissa,1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Guwahati,4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+
Pune,6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+
Delhi,4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mumbai,1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Surat,3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Rohtak,1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Chennai,5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Kolkata 10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+
Bihar,2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Fig. 1. Amplification of *ctxB* gene in 45 strains with biotype specific primers. (Upper panel) PCR with Re-cla primers showing 186 bp PCR product. From left O395 (classical control), N16961 El Tor control), 1-VOC1, 2-G1, 3-P3, 4-M24, 5-AFMC1, 6-IDH00563, 7-IDH00734, 8-IDH00725, 9-B1, 10-S4, 11-VOC27, 12-VOC30, 13-SRK1, 14-IDH00614, 15-4750. (Lower panel) PCR with Rv-elt primers. From left O395 (classical control), N16961 (El Tor control), 1-VOC1, 2-G1, 3-P3, 4-M24, 5-AFMC1, 6-IDH00563, 7-IDH00734, 8-IDH00725, 9-B1, 10-S4, 11-VOC27, 12-VOC30, 13-SRK1, 14-IDH00614, 15-4750. (Lower panel) PCR with Rv-elt primers.

Rv-cla [14] that was exclusively designed to detect the single base change at the 58th nucleotide position [14] that was originally found in strains associated with a recent outbreak of cholera in Orissa [24].

DNA sequencing of the 460 bp *ctxB* gene was carried out for comparing the sequence of nucleotides in these two groups of strains at the positions already designated to contain SNPs [16] and also for the confirmation of the results of D-MAMA PCR in this study. Sequence analysis revealed that all the strains had identical *ctxB* gene sequence like the classical standard control O395 with cytosine (C) at positions 115 and 203 [16] However, in the 16 strains that showed positive amplification result with D-MAMA PCR an expected base change was identified at the 58th nucleotide position wherein, the cytosine (C) at this position was found to be replaced with adenine (A) resulting in a change in the deduced amino acid sequence. Consequently, asparagine had replaced histidine at the 20th amino acid position (Table 4).

Beads ELISA and comparison of CT production

Beads ELISA [18] showed that these strains produce CT in the range of 0 to 2774 ng. The replacement of
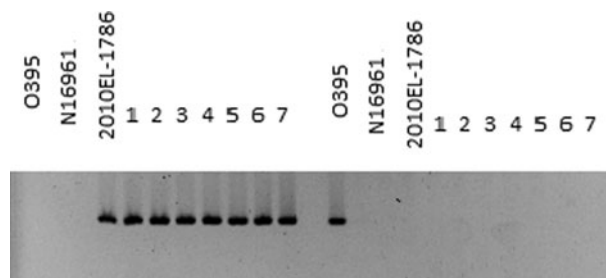
Fig. 2. D-MAMA PCR with 7 representative strains. (Lanes from left): O395 (Classical control), N16961 (El Tor control), 2010EL-1786 (Haitian control), 1-IDH00563 (Kolkata, 2008), 2-IDH00725 (Kolkata, 2008), 3-IDH00734 (Kolkata, 2008), 4-VOC30 (Orissa, 2007), 5-VOC27 (Orissa, 2007), 6-P7 (Pune, 2007), 7-AFMC1 (Pune, 2008), Blank well, O395 (Classical control), N16961 (El Tor control), 2010EL-1786 (Haitian control), 1-IDH00563 (Kolkata, 2008), 2-IDH00725 (Kolkata, 2008), 3-IDH00734 (Kolkata, 2008), 4-VOC30 (Orissa, 2007), 5-VOC27 (Orissa, 2007), 6-P7 (Pune, 2007), 7-AFMC1 (Pune, 2008). Left side of the blank well PCR done, using *ctxB* F3/Rv-cla primer. Right side of the blank well PCR done, using *ctxB* F4/Rv-cla primer.

histidine with asparagine at the 20th amino acid position in the CTB sequence of the 16 strains did not lead to any significant change associated with the amount of CT production as compared with the strains with amino acid histidine at this position.

Comparison of different clones by PFGE

PFGE was used to determine clonality of seven representative strains. From the dendogram that was constructed four distinct profiles, PI-PIV were observed among these seven strains (Fig. 3).

These four major clusters were designated as PFI, PFII, PFIII and PFIV existed among the seven strains selected for the dendogram construction (Fig. 4). Approximately 97% similarity was found between PFI and PFII, 95% between PFII and PFIII and 92% between PFIII and PFIV.

All of these seven strains had uniform phenotypic and genetic characteristics but still belonged to distinct PFGE profiles.

DISCUSSION

Cholera remains a public health threat in cholera endemic areas in India [25]. Among the 36 states and union territories almost every Indian state has witnessed outbreaks of cholera in the recent past. West Bengal, Orissa, Assam, Chattisgarh and the

Table 4. *Places in India from where* ctxB *genotype 7 has been isolated by* ctxB *sequencing of strains that caused cholera in these places in the years 2007 and 2008*

Place, Year	*ctxB* genotype
Guwahati, 2007	1,7
Orissa, 2007	7
Orissa, 2008	1
Mumbai, 2008	1
Pune, 2007	7
Pune, 2008	7
Rohtak, 2007	1
Surat, 2008	1
Surat, 2008	1
Chennai, 2007	1
Chennai, 2008	1
Delhi, 2007	1
Delhi, 2008	1
Bihar, 2008	1
Kolkata, 2007	1
Kolkata, 2008	1,7

Fig. 3. PFGE gel showing four distinct NotI digestion profiles among 7 representative strains isolated from India in 2007 and 2008. Lanes-1-*S. enterica* serovar Braenderup strain H9812 (Marker), 2-VOC27, 3-VOC30, 4-P7, 5-AFMC1, 6-*S.enterica* serovar Braenderup strain H9812 (Marker), 7-IDHOO563, 8-IDH00725, 9-IDHOO734, 10-*S.enterica* serovar Braenderup strain H9812 (Marker).

union territory of Andaman and Nicobar Islands reported the highest number of cases, accounting for 91%, according to a surveillance related study conducted over a period of 10 years [26]. In addition, underreporting overshadows actual number of cholera cases in India leading to discrepancies in the incidence of cholera [27].

At present, altered El Tor strains that were identified from isolates from 1993 onwards and replaced the prototype El Tor strains globally since 2001 [8] continue to cause cholera outbreaks in the country. Due to increasing mortality and morbidity caused by the pathogen quick epidemiological investigations based on molecular typing techniques is the need of the hour for rapid administration of control and preventive measures during an outbreak. Also, molecular typing methods are applied to resolve any ambiguity that may arise in the process of determining the biotype of the isolates. For example, previous studies showed the existence of strains like the Mozambique strains that are hybrids between the two biotypes and carry classical CTXφ and also unique El Tor genes like VCA0728-VCA0730 and phenotypic properties of the El Tor biotype [28]. In such contexts, molecular typing is highly advantageous.

This retrospective genetic analysis using molecular techniques is a valuable study that has compared genetic features of *V. cholerae* strains from diverse locations in India unlike other studies with India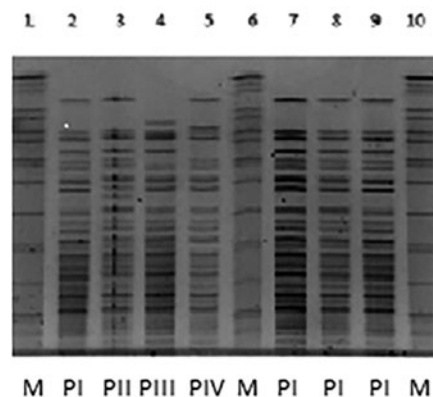n strains that have a more limited geographical coverage. It is a continuation of a previous study by Naha *et al.* [14] and has shown that the strains with *ctxB-7* allele did not remain confined in terms of geographical distribution to Eastern India in Kolkata [14] or Orissa [24] but were circulating in different cholera endemic regions in the country.

These strains have a single copy of CTXφ in the genome. Difference in arrangement of the CTXφ in the chromosome exists between classical and El Tor biotypes [28]. Recent findings report the presence of two copies of the *ctxB* gene, one on each chromosome contrary to previous reports that *ctxAB* genes are located only in the large chromosome in El Tor biotype [29]. Recent studies on El Tor variant strains from an outbreak in Hyderabad, India showed that one particular strain VCH35 carries two copies of the classical CTXφ in tandem repeat in the small chromosome [30]. However, the organization and copy number of the CTXφ in the strains included in this study is identical to that of prototype El Tor strains [31]. Also, these strains possess unique genes of the El Tor biotype. Classical and El Tor biotypes arose from different ancestors [32] and vary distinctly in their variable genome [12]. Profiling of virulence genes can be used to trace the lineage of strains [33] thus helping in biotype identification. The genetic variation observed among these strains did not affect CT production nor had any correlation with PFGE profiles.
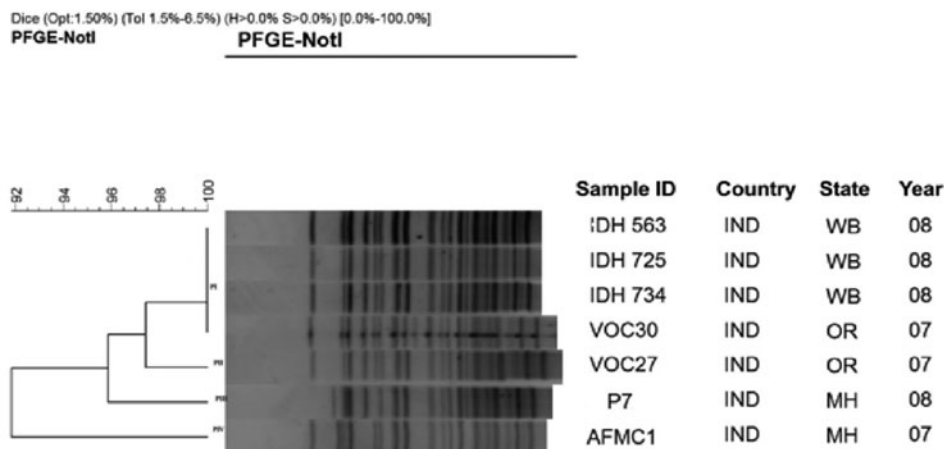
Dice (Opt:1.50%) (Tol 1.5%-6.5%) (H>0.0% S>0.0%) [0.0%-100.0%]
PFGE-NotI   PFGE-NotI

Fig. 4. Dendogram showing clonal relationship among representative strains.

This study has also enabled one to get a glimpse into the genetic composition of Indian strains from the same period that is a milestone in the evolutionary history of the pathogen in several ways when the first reports of novel *ctxB-7* allele [24] and other crucial genetic changes had gradually started appearing in the contemporary *V. cholerae* strains worldwide as reported by several investigators [34, 35].

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