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# Molecular Characterization of *Vibrio cholerae* Isolates from Cholera Outbreaks in North India

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***Vibrio cholerae*** **isolates recovered from cholera outbreaks in Bhind district of Madhya Pradesh and Delhi, Northern India were characterized. The O1 serogroup isolates from Bhind outbreak were of Inaba serotype whereas both Ogawa and Inaba serotypes were recovered from Delhi. PCR analysis revealed that only O1 serogroup *V. cholerae* isolates carried the virulence-associated genes like *ctxA*, *tcpA*, *ace*, and *zot*. Molecular typing by repetitive sequence based ERIC, VCR1, and VC1 PCR's revealed similar DNA profile for both Inaba and Ogawa serotypes. A discrete VC1-PCR band identified among the El Tor strains had greater similarity (>97%) to the *V. cholerae* genome sequence and therefore has the potential to be used as a marker for the identification of the *V. cholerae* strains. Non-O1 strains recovered from Bhind region differed among themselves as well as from that of the O1 isolates. All the O1 serogroup isolates possessed SXT element and were uniformly resistant to the antibiotics nalidixic acid, polymyxin-B, furazolidone, cloxacilin, trimethoprim-sulfamethaxazole, and vibriostatic agent O129. Inaba strains from both Delhi and Bhind differed from Ogawa strains by their resistance to streptomycin despite sharing similar DNA patterns in all the three rep-PCRs. Though Delhi and Bhind are separate geographical regions in Northern India, Inaba strains from both these places appear to be closely related owing to their similarity in antibiogram and genetic profile.**

***Keywords*****:** ERIC-PCR, DNA fingerprinting, SXT, VC1-PCR, *Vibrio cholerae*

Cholera is a severe infectious diarrheal disease caused by toxigenic *Vibrio cholerae*. The disease is characterized by stools of rice water diarrhea that rapidly leads to dehydration. Cholera has the potential to appear in explosive outbreaks, often emerging in distinct geographical locations simultaneously or with sufficient temporal gap. It can also cause true pandemics that severely affect many countries in multiple continents over a span of many years (Kaper *et al*., 1995). In the Indian sub-continent, the incidence pattern of *V. cholerae* O1 serogroup has been changing constantly with the predominance of Inaba or Ogawa serotype of El Tor (Garg *et al*., 2000; Sur *et al*., 2006).

Molecular techniques such as ribotyping, RFLP of *ctx* gene, multilocus enzyme electrophoresis and other PCR based DNA fingerprinting methods produce strain specific patterns that aid in strain level differentiation of the epidemic isolates. They are widely used to study relationship among isolates from outbreaks as well as from the environment. Among all these, PCR based DNA fingerprinting methods based on repetitive sequences are easy to perform, consume very less time, and correlate well with ribotyping. Use of specific primers directed towards the conserved region in these interspersed repetitive elements and the stringent conditions employed in the PCR assays enable promising DNA fingerprints and can overcome the standardisation problems and lack of reproducibility associated with RAPD (Olive and Bean, 1999). A combination of molecular markers based on repetitive sequences would bring out the exact polymorphism present among the isolates (Woods *et al*., 1993; Shangkuan *et al*., 1997b). Characteristic prokaryotic repeats such ERIC, REP, and BOX have been reported in numerous eubacterial members and primers derived from these interspersed repeats have been employed in molecular typing of bacterial strains (Belkum *et al*., 1998). The VC1 primer derived from ERIC related sequence in *V. cholerae* and VCR1 primer designed from a repeat sequence associated with a locus encoding a haemagglutinin in *V. cholerae* have been used for molecular typing of *V. cholerae* strains elsewhere (Shangkuan *et al*., 1997a).

*V. cholerae* isolates were recovered from the cholera outbreaks in Bhind district of Madhya Pradesh (19 isolates) and Delhi (20 isolates) during May and August 2004 respectively. Preliminary analysis of the isolates was done by employing conventional biochemical testing, serotyping, and antimicrobial susceptibility testing. Molecular characterization was performed by screening the isolates for the presence of conserved *ompW* gene, SXT element and epidemiologically important virulent genes like *ctxA*, *tcpA*, *ace*, and *zot*. DNA fingerprinting by repetitive sequence based molecular markers like ERIC, VC1, and VCR1 PCRs was carried out subsequently to study the strain level differences.

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## Materials and Methods

### Bacterial strains

Stool, vomit samples or rectal swabs were collected from patients with diarrhea admitted to the district hospital, Bhind. Sterile swabs dipped in stool/vomit samples and the rectal swabs collected from the patients were placed in Cary Blair transport media and transported to the lab within 3 h time. Surface water of 100 ml each, collected from water bodies and hand pumps in the affected areas of Bhind district were passed through sterile membrane filter (pore size 0.22 μm, Millipore, USA, using a filtration manifold) and the filters were used to inoculate alkaline peptone water (APW). Five-hour incubation at 37°C was given and the surface growth was streaked onto thiosulphate citrate bile salt (TCBS) agar (Difco, USA) plates. A single colony of the cultures that produced bright yellow color was inoculated in Gelatin agar (Hi-Media, India) plates after an APW passage. Three reference strains, one each of El Tor biotype, O1 Classical biotype and O139 serogroup obtained from NICED, Calcutta were also used for comparison. All the isolates were examined for gelatinase activity and the isolates that produced a halo in Gelatin agar plates were checked for oxidase reaction. The oxidase positive isolates were then tested for the darting motility under 40× low power lens, decarboxylase/dihydrolase activity of amino acids Arginine, Lysine, and Ornithine. The isolates that were positive for *V. cholerae* by biochemical characterization were subjected to slide agglutination with Polyvalent and Monovalent O1 antisera obtained from Difco.

### Antibiotic susceptibility analysis

Antimicrobial susceptibility analysis of O1 *V. cholerae* strains was performed by disk diffusion method (Bauer *et al*., 1966) on Muller Hinton agar (Difco) using antibiotic discs (Oxoid Ltd., UK) containing tetracycline (Te) 30 μg, amikacin (Ak) 30 μg, amoxycilin (Aml) 10 μg, chloramphenicol (Cl) 30 μg, cephalexin (C) 30 μg, ciprofloxacin (Cip) 5 μg, cloxacilin (OB) 5 μg, gentamycin (CN) 10 μg, erythromycin (E) 15 μg, kanamycin (K) 30 μg, norfloxacin (Nor) 10 μg, penicillin G (P) 10 μg, ampicillin (Amp) 10 μg, nalidixic acid (Na) 30 μg, polymyxin-B (Pb) 300 U, streptomycin (S) 10 μg, furazolidone (Fr) 100 μg, trimethoprim-sulfamethaxazole (Sxt) 25 μg, and vibriostatic agent O129 (DD15). The isolates were scored as sensitive or resistant as per the CLSI guidelines (Clinical and Laboratory Standards, 2005).

### PCR assays

The *V. cholerae* strains grown overnight at 37°C in LB broth (Hi-Media) were boiled and stored at -20°C for further use. One microliter of the lysate was used as template for the PCR assays. *V. cholerae* strains were examined for the presence of *ompW*, *ctxA*, *tcpA*, *ace*, *zot* genes and SXT element. Presence of *ompW* and *ctxA* genes was determined by the duplex PCR assay described previously (Nandi *et al*., 2000). Primers used in the duplex PCR assay and other PCR reactions; their annealing conditions and the size of amplicons obtained are shown in the Table 1. PCR was per-

**Table 1.** Primers used in this study to detect *omp W*, virulence, and SXT genes in *V. cholerae*

| Genes | Primer sequences (5' to 3') | Amplicon size (bp) | Annealing conditions | References |
|---|---|---|---|---|
| *omp W*-F | CACCAAGAAGGTGACTTTATTGTG | 588 | 64°C, 30 sec | Nandi *et al*. (2000) |
| *omp W*-R | GAACTTATAACCACCCGCG | | | |
| *ctxA*-F | CTCAGACGGGATTTGTTAGGCACG | 301 | 64°C, 1 min | Nandi *et al*. (2000) |
| *ctxA*-B | TCTATCTCTGTAGCCCCTATTACG | | | |
| *tcpA*-F | CACGATAAGAAAACCGGTCAAGAG | 453 | 62°C, 1 min | Rivera *et al*. (2001) |
| *tcpA*-B/Clas | TTACCAAATGCAACGCCGAATG | | | |
| *tcpA*-B/[a] | CGAAAGCACCTTCTTTCACACGTTG | | | |
| *ace*-F | TAAGGATGTGCTTATGATGGACACCC | 316 | 62°C, 1 min | Shi *et al*. (1998) |
| *ace*-B | CGTGATGAATAAAGATACTCATAGG | | | |
| *zot*-F | TCGCTTAACGATGGCGCGTTTT | 947 | 62°C, 1 min | Rivera *et al*. (2001) |
| *zot*-B | AACCCCGTTTCACTTCTACCCA | | | |
| SXTf | ATGGCGTTATCAGTTAGCTGGC | 1035 | 58°C, 1 min | Bhanumathi *et al*. (2003) |
| SXTr | GCGAAGATCATGCATAGACC | | | |
| ERIC 1R | ATGTAAGCTCCTGGGGATTCAC | | | Rivera *et al*. (1995) |
| ERIC 2 | AAGTAAGTGACTGGGGTGAGCG | | | |
| VC1 | AACTGTGTGATTAGGATGAACGAA | | | Shangkuan *et al.* (1997a) |
| VCR1 | CGCGTTGACAGTCCCTCTTGA | | | Shangkuan *et al.* (1997a) |

[a] ElTor/O139

**Table 2.** Results of serotyping, antibiotic susceptibility tests, and PCR assays

| *V. cholerae* isolates | | *V. cholerae* isolates positive for | | Serotyping | Antibiogram |
|---|---|---|---|---|---|
| Place | Serogrouping | *omp W* | Virulence genes[c] | | |
| Bhind[a] | O1 (Clin) | 13 | 13 | Inaba-13 | $NA^R$, $PB^R$, $S^R$, $Fr^R$, $OB^R$, $SXT^R$, $DD15^R$ |
| | Non O1, Non O139 (Clin) | 2 | 0 | - | - |
| | Non O1, Non O139 (Envt) | 4 | 0 | - | - |
| Delhi[b] | O1 (Clin) | 20 | 20 | Inaba-12 | $Na^R$, $Pb^R$, $S^R$, $Fr^R$, $OB^R$, $SXT^R$, $DD15^R$ |
| | | | | Ogawa-8 | $Na^R$, $Pb^R$, $S^S$, $Fr^R$, $OB^R$, $SXT^R$, $DD15^R$ |

[a] A total of 15 Clinical isolates & 4 Envt isolates analyzed
[b] A total of 20 Clinical isolates analyzed
[c] *ctxA*, *tcpA*, *ace*, and *zot*

formed in a reaction volume of 20 μl with 0.5 μM each forward and reverse primers, 2.0 μl of 10× PCR buffer, 1 mM of $MgCl_2$ (final conc.), 100 μM each of dATP, dCTP, dTTP, dGTP and 0.5 U of *Taq* polymerase using the following program on an *i*-cycler system (BioRad laboratories Inc., USA): an initial denaturation (95°C for 3 min), followed by 30 cycles of denaturation (94°C for 30 sec), annealing for 1 min (Table 1) and extension (72°C for 1 min). A final extension of 72°C for 6 min was given at the end of every PCR reaction. The amplified products were subjected to agarose (0.8% w/v) gel electrophoresis in Tris-Borate EDTA (0.5× TBE), stained in ethidium bromide and visualized using Imagemaster VDS gel documentation system (Amersham-Pharmacia Biotech AB, Sweden).

## Confirmations of the amplicons by Dot blot hybridization (DBH)

Dot blots were prepared for the individual genes separately using the respective PCR amplicons. Ten microliter of PCR product was denatured by boiling in 90 μl of 10× SSC for 10 min and transferred to ice for 5 min. The denatured product was then loaded onto Hybond N+ nylon membrane (pre-soaked in 10× SSC solution for 30 min) using a dot blot apparatus (Biodot, BioRad laboratories Inc., USA). The membrane was air dried and fixed by UV cross-linker (UVP). The probes were prepared by PCR amplification of target genes *ompW*, *ctxA*, *tcpA*, *ace*, and *zot* using the El Tor DNA as the template. Appropriate amplicons, thus obtained were purified by using the QIAQuick purification kit (QIAGEN GmbH, Germany) and labelled with digoxigenin-UTP using DIG DNA labelling and detection kit (La Roche, France). Membranes were prehybridized followed by overnight hybridisation at 42°C in DIG Easy Hyb buffer (La Roche) with their respective probes and the positive hybridisations read visually by enzyme immunoassay as described by the manufacturer.

## ERIC, VC1, and VCR1 PCRs

PCR DNA fingerprinting by ERIC, VC1, and VCR1 primers was carried out in a final volume of 20 μl using the primers and temperature ramping as described by Shangkuan (1997a). The reaction mixture contained 1 μl of template DNA, 2 μl of 10× polymerase reaction buffer, 3 mM $MgCl_2$ (final conc), 200 μM each of (final conc) dATP, dCTP, dTTP, and dGTP, 1.5 U *Taq* polymerase, the primers (100 ng each) ERIC-1R and ERIC-2 for ERIC-PCR, primers VC1, VCR1 for VC1-PCR, and VCR1-PCR, respectively. For every PCR reaction, a negative control (no template DNA) and a positive control (template DNA giving amplified product) were maintained. The amplified products were separated by agarose (1% w/v) gel electrophoresis in Tris-Borate EDTA (0.5× TBE), stained in ethidium bromide and visualized using Imagemaster VDS gel documentation system (Amersham-Pharmacia Biotech AB).

## Cloning and sequencing of the VC1-PCR marker

For DNA sequencing, the selected 1,400 bp VC1-PCR marker from VcB28 was cloned into the linear plasmid vector pDRIVE supplied in the QIAGEN PCR cloning kit (Germany, GmbH), according to the manufacturer's protocol. Cloned fragments were custom sequenced (MWG Biotech) using M13 reverse primer. The GenBank accession number assigned for the sequence submitted is FJ360752. The BLAST (Basic Local Alignment Search Tool) program was

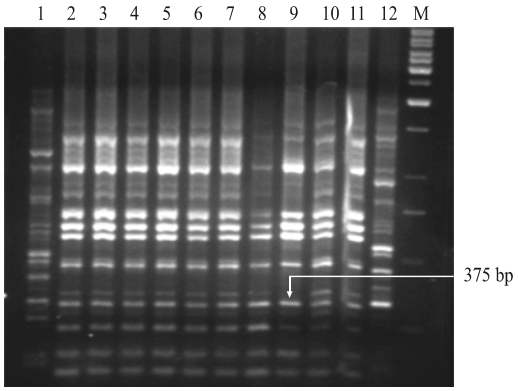


**Fig. 1.** *V. cholerae* genotypes identified using ERIC primers. The O1 isolates obtained from Bhind district have the prefix VcB, those from Delhi VcD, and those from the environment VcE. As all the O1 *V. cholerae* isolates from both the outbreaks showed the similar DNA patterns, few isolates are shown in the illustration. O1 El Tor, O1 Classical, and O139 are standard strains from NICED. [A]Ogawa serotypes, [B]Inaba serotypes analyzed in this study. Lanes: M, 1 kb ladder; 1, VcB1; 2, $VcB28^B$; 3, $VcB29^B$; 4, $VcB32^B$; 5, $VcD1^A$; 6, $VcD2^A$; 7, $VcD7^B$; 8, $VcD11^B$; 9, O1Classical; 10, O139; 11, El Tor; 12, VcB26.

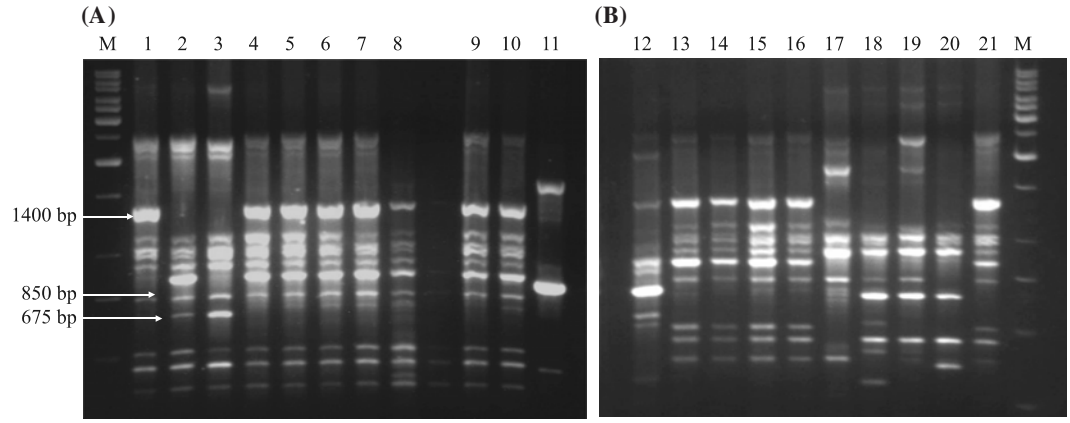


**Fig. 2.** *V. cholerae* genotypes identified using VC1 primers. Few isolates with similar DNA fingerprints were not shown. [A]Ogawa serotypes, [B]Inaba serotypes analyzed in this study. Lanes: M, 1 kb ladder; 1, El Tor; 2, O139; 3, O1 Classical; 4, VcD1[A]; 5, VcD2[A]; 6, VcD6[A]; 7, VcD7[B]; 8,VcD11[B]; 9, VcD14[B]; 10, VcD19[B]; 11, VcB1; 12, VcB26; 13, VcB28[B]; 14, VcB29[B]; 15, VcB32[B]; 16, VcB33[B]; 17, VcE22; 18, VcE35; 19, VcE36; 20, VcE37; 21, VcB6[B].

used to conduct similarity searches against the GenBank sequence database.

## Results

A total of 39 *V. cholerae* isolates confirmed by biochemical tests were used in the study. This included 15 isolates from patients of Bhind outbreak, 20 isolates from Delhi outbreak and 4 isolates from the water sources of affected areas in Bhind District. All the isolates showed darting motility, oxidase positive and could decarboxylate Lysine and Ornithine. A negative reaction was seen with respect to arginine dihydrolase. Serotyping with polyvalent O1 antisera showed that 33 clinical isolates belonged to O1 serogroup whereas, 2 clinical and 4 environmental isolates were of non-O1 serogroup. The O1 isolates from Bhind were identified to be Inaba serotype whereas both Inaba (60%) and Ogawa (40%) serotypes were recovered from Delhi. The O1 *V. cholerae* isolates were uniformly sensitive to the antibiotics, amikacin, amoxycilin, chloramphenicol, cephalexin, ciprofloxacin, gentamycin, erythromycin, kanamycin, norfloxacin, penicillin G, tetracycline, ampicillin, and resistant to nalidixic acid, polymyxin-B, furazolidone, cloxacilin, trimethoprim-sulfamethaxazole, and vibriostatic agent O129. The Inaba strains differed from Ogawa strains by their resistance to streptomycin.

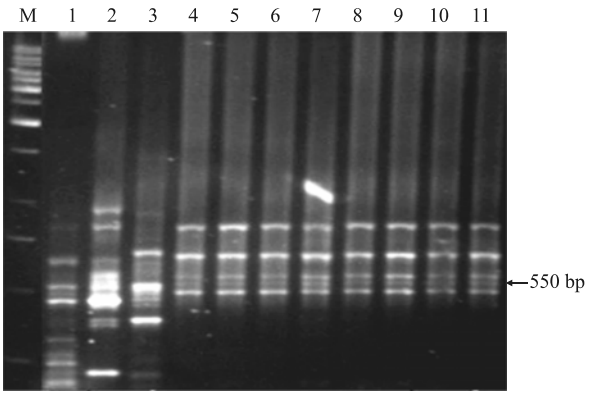


**Fig. 3.** *V. cholerae* genotypes identified using VCR1 primers. Lanes: M, 1 kb ladder; 1, VcE 22; 2, VcE 35; 3, VcE 37; 4, VcB28[B]; 5, VcB29[B]; 6, VcB32[B]; 7, VcB33[B]; 8, VcD1[A]; 9, VcD2[A]; 10, VcD7[B]; 11, VcD11[B]; [A]Ogawa serotypes, [B]Inaba serotypes analyzed in this study.

All the clinical and environmental isolates were found to be positive for *ompW* gene and produced an amplicon of about 588 bp. Thirty-three clinical isolates of O1 serogroup were found to possess *ctxA*, *tcpA*, *ace*, and *zot* genes and produced amplicons of 301 bp, 453 bp, 316 bp, and 947 bp, respectively. The dig-labelled probes hybridised with all their respective positive PCR amplicons thereby confirming the identity of the individual PCR amplicons. All the O1 isolates produced positive PCR results (1,035 bp band) with SXT primers. The isolates used in the study, results of serotyping, antibiotic susceptibility tests, and PCR assays are given in Table 2.

DNA fingerprinting with ERIC-PCR revealed about 34 different fragments of DNA ranging from about 0.1 kb to 2.1 kb (Fig. 1). The reference O1 Classical strain could be differentiated from other toxigenic strains by the absence of a 375 bp fragment while the reference O139 and O1 El Tor strains produced identical profiles. The non-toxigenic *V. cholerae* isolates from clinical as well as environmental samples showed distinct profiles, which clearly differentiated them from the toxigenic isolates of O1 and O139 serovars. 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. 2A and B). All the 3 reference strains showed different patterns with this primer. The El Tor reference strain and other El Tor isolates used in this study showed a prominent band of 1,400 bp, which was absent in O1 Classical and O139 strains. The 741 bp region sequenced out of the discreet 1,400 bp VC1-PCR band had high level of identity (97%) only with the *V. cholerae* whole genome sequence CP000627 (position 1994018-

1994748) and AE003852 (position 2440676-2441406). A prominent band of about 675 bp was present in O1 Classical and O139 strains and not in other toxigenic strains. Representative DNA patterns obtained with VCR1 primer are given in Fig. 3. The DNA fragments of about 550 bp and 575 bp seen in the case of the toxigenic O1 isolates were absent in any of the reference strains.

## Discussion

The *V. cholerae* strains isolated from the cholera patients in Bhind and Delhi were characterized in detail to examine the presence of virulence traits, SXT constin and their genetic relatedness. The *ompW* gene, which is normally targeted for the species-specific identification of *V. cholerae* (Nandi *et al*., 2000) was present among all the 39 *V. cholerae* isolates studied. *TCP A* gene, a major component of TCP gene cluster is found to exhibit nucleotide sequence difference between El Tor O1/O139 and Classical strains and is employed for the biotyping purposes. Thirty-three *V. cholerae* strains that showed positive agglutination with the polyvalent O1 antisera revealed a 453 bp amplicon with the *tcpA* PCR assay that helped their confirmation as El Tor strains. These O1 El Tor strains were also positive for *ctxA*, *tcp*A, *ace*, *zot*, genes indicating that the major genes which are important components of the CTX element and TCP pathogenicity island required for the pathogenesis were present in O1 El Tor strains whereas, the non O1 strains isolated during this study were negative for these genes and therefore, assumed to be non-toxigenic.

The primers based on 3 different types of repetitive sequences that are associated with different genes and evenly scattered in the chromosome viz, ERIC, VC1, VCR1 were employed for DNA fingerprinting. Their position in the chromosome may vary and the DNA profiles from each primer set would present the polymorphism present in different chromosomal loci among the isolates. Among the rep-PCR molecular markers used, ERIC was less polymorphic as it could not differentiate the El Tor and O139 reference strains. This may be due to the fact that O139 serogroup is said to have evolved from El Tor and may be very closely related to it (Faruque *et al*., 2003). Earlier studies using a single ERIC primer (ERIC IR) could not reveal the variation present between the toxigenic-Classical and El Tor strains (Shangkuan *et al*., 1997a). In the present study the toxigenic Classical and El Tor strains could be differentiated when both the primers, ERIC IR and ERIC2 were used. Further VCR1 and VC1 rep-PCRs could reveal the genetic variation between the toxigenic strains of O1 and O139 serogroups and also that between the Classical and El Tor biotypes of O1 *V. cholerae* and hence served as polymorphic molecular markers. *V. cholerae* specific sequence identified out of the 1,400 bp VC1-PCR band has the potential to be developed to a specific marker for the detection of *V. cholerae.* However, further study is needed to test its specificity against the different serogroups and other closely related species of *V. cholerae*.

Water samples obtained from surface water bodies and hand pumps from the affected regions of Bhind District during the outbreak period contained *V. cholerae*, but were found to be non-toxigenic/non O1 strains and they showed entirely different DNA fingerprints with respect to ERIC, VC1 and VCR1 primers. The non-O1 isolates were shown to exhibit considerable diversity even by the less polymorphic ERIC primer. The reason for this greater genetic diversity may be due to genetic reassortments these isolates undergo during their survival in the environment (Dalsgaard *et al*., 1999).

The Inaba strains differed from the Ogawa strains obtained from Delhi by their resistance to streptomycin. Both the serotypes revealed the presence of SXT element that is considered to be associated with the spread of genetic determinants for the resistance to antimicrobial agents in the *V. cholerae* strains isolated during recent years. Even though SXT elements are known to hold the genetic determinants for chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole and streptomycin resistance (Amita *et al*., 2003), there is considerable flux in the antibiotic resistance genes found in the SXT (Hochhut *et al*., 2001; Iwanaga *et al*., 2004; Ramachandran *et al*., 2007). The flux in the antibiotic resistance genes present in the SXT constin may be the reason for the variation in streptomycin resistance among these El Tor strains. All the isolates recovered from the present outbreaks were found to be resistant to more than 4 antibiotics and hence they can also be termed as multiple antibiotic resistant (MAR) (Kondo *et al*., 1995). These MAR strains showed resistance against the antibiotics nalidixic acid, polymyxin-B, cloxacilin, trimethopim-sulfamethaxazole, furazolidone, vibriostatic compound-agent O129, and streptomycin.

Even though the O1 *V. cholerae* strains recovered from these outbreaks were resistant to the antibiotics furozolidone and trimethoprim-sulfamethoxazole which were widely prescribed earlier, they were fortunately susceptible to norfloxacin, ciprofloxacin, and tetracycline that stay to be the main adjuncts of antibiotic therapy for cholera nowadays.

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## References

Amita, S., R. Chowdhury, M. Thungapathra, T. Ramamurthy, G.B. Nair, and A. Ghosh. 2003. Class I Integrons and SXT Elements in El Tor Strains Isolated before and after 1992 *Vibrio cholerae* O139 Outbreak, Calcutta, India. *Emerg. Infect. Dis.* 9, 500-502.

Bauer, A.W., W.M. Kirby, J.C. Sherris, and M. Truck. 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* 45, 493-496.

Belkum, A.V., S. Scherer, L.V. Alphen, and H. Verbrugh. 1998. Short-sequence repeat in prokaryotic genomes. *Microbiol. Mol. Biol. Rev.* 62, 275-293.

Bhanumathi, R., F. Sabeena, S.R. Isac, B.N. Shukla, and D.V. Singh. 2003. Molecular characterization of *Vibrio cholerae* O139 bengal isolated from water and the aquatic plant *Eichhornia crassipes* in the River Ganga, Varanasi, India. *Appl. Environ. Microbiol.* 69, 2389-2394.

Clinical and Laboratory Standards. 2005. Performance standards for antimicrobial susceptibility testing. Fifteenth informational supplement. Clinicial and Laboratory Standards Institute. Wayne, Pa, USA; M100-S15.

Dalsgaard, A., A. Forslund, L. Bodhidatta, O. Serichantalergs, C. Pitarangsi, L. Pang, T. Shimada, and P. Echeverria. 1999. A high proportion of *Vibrio cholerae* strains isolated from children with diarrhea in Bangkok, Thailand are multiple antibiotic resistant and belong to heterogenous non-O1, non-O139 O-serotypes. *Epidemiol. Infect.* 122, 217-226.

Faruque, S.M., D.A. Sack, R.B. Sack, R.R. Colwell, Y. Takeda, and G.B. Nair. 2003. Emergence and evolution of *Vibrio cholerae* O139. *Proc. Natl. Acad. Sci. USA* 100, 1304-1309.

Garg, P., R.K. Nandy, P. Chaudhury, N.R. Chowdhury, K. De, T. Ramamurthy, S. Yamasaki, S.K. Bhattacharya, Y. Takeda, and G.B. Nair. 2000. Emergence of *Vibrio cholerae* O1 biotype El Tor serotype Inaba from the prevailing O1 Ogawa serotype strains in India. *J. Clin. Microbiol.* 38, 4249-4253.

Hochhut, B., Y. Lotfi, D. Mazel, S.M. Faruque, R. Woodgate, and M.K. Waldor. 2001. Molecular analysis of antibiotic resistance gene clusters in *Vibrio cholerae* O139 and O1 SXT constins. *Antimicrob. Agents Chemother*. 45, 2991-3000.

Iwanaga, M., C. Toma, T. Miyazato, S. Insisiengmay, N. Nakasone, and M. Ehara. 2004. Antibiotic resistance conferred by a class I integron and SXT constin in *Vibrio cholerae* O1 strains isolated in Laos. *Antimicrob. Agents Chemother.* 48, 2364-2369.

Kaper, J.B., J.G. Morris, and M.M. Levine. 1995. Cholera. *Clin. Microbiol. Rev.* 8, 48-86.

Kondo, S., U. Kongmuang, S. Kalnauwakul, C. Matsumoto, C.H. Chen, and M. Nishibuchi. 1995. Molecular epidemiologic analysis of *Vibrio cholerae* O1 isolated during the 1997-8 cholera epidemic in southern Thailand. *Epidemiol. Infect.* 114, 51-63.

Nandi, B., R.K. Nandy, S. Muhopadhyay, G.B. Nair, T. Shimada, and A.C. Ghose. 2000. Rapid method for species-specific identification of *Vibrio cholerae* using primers targeted to the gene of outer membrane protein Omp W. *J. Clin. Microbiol.* 38, 4145-4151.

Olive, D.M. and P. Bean. 1999. Principles and applications of methods for DNA-based typing of microbial organisms. *J. Clin. Microbiol.* 37, 1661-1669.

Ramachandran, D., R. Bhanumathi, and D.V. Singh. 2007. Multiplex PCR for detection of antibiotic resistance genes and the SXT element: application in the characterization of *Vibrio cholerae*. *J. Med. Microbiol*. 56, 346-351.

Rivera, I.N.G., J. Chun, A. Huq, R.B. Sack, and R.R. Colwell. 2001. Genotype associated with virulence in environmental isolates of *Vibrio cholerae. Appl. Environ. Microbiol.* 67, 2421-2429.

Shangkuan, Y.H., H.C. Lin, and T.M. Wang. 1997a. Diversity of DNA sequences among *Vibrio cholerae* O1 and non-O1 isolates detected by whole-cell repetitive element sequence based polymerase chain reaction. *J. Appl. Microbiol.* 82, 335-344.

Shangkuan, Y.H., C.M. Tsao, and H.C Lin. 1997b. Comparison of *Vibrio cholerae* isolates by polymerase chain reaction fingerprinting and ribotyping. *J. Med. Microbiol.* 46, 941-948.

Shi, L., S. Miyoshi, M. Hiura, K. Tomochika, T. Shimada, and S. Shinod. 1998. Detection of genes encoding cholera toxin (CT), zonula occludens toxin (ZOT), accessory cholera enterotoxin (ACE) and heat stable enterotoxin (ST) in *Vibrio mimicus* clinical strains. *Microbiol. Immunol.* 42, 823-828.

Sur, D., B.L. Sarkar, B. Manna, J. Deen, S. Datta, S.K. Niyogi, A.N. Ghosh, A. Deb, S. Kanungo, A. Palit, and S.K. Bhattacharya. 2006. Epidemiological, microbiological & electron microscopic study of a cholera outbreak in a Kolkata slum community. *Indian J. Med. Res.* 123, 31-36.

Woods, C.R., J. Versalovic, T. Koeuth, and J.R. Lupski. 1993. Whole-cell repetitive element sequence based polymerase chain reaction allows rapid assessment of clonal relationships of bacterial isolates. *J. Clin. Microbiol.* 31, 1927-1931.