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# Molecular epidemiology of *Vibrio cholerae* causing outbreaks & sporadic cholera in northern India

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***Background & objectives*: Several outbreaks of cholera have been reported in Chandigarh region during a span of seven years from 2002-2008. The genetic characteristics of *Vibrio cholerae* isolates obtained during these outbreaks have not been adequately studied. The aim of this study was to do molecular typing of *V. cholerae* isolated from the sporadic and outbreak cases by pulsed-field gel electrophoresis (PFGE), Rep-PCR and ribotyping.**

***Methods*: Fifty representative isolates of *V. cholerae* from outbreak as well as sporadic cases were subjected to molecular typing by PFGE, 173 isolates (163 clinical and 10 environmental) were typed by rep-PCR and ribotyping. Ribotyping was done by determination of rRNA restriction pattern of *Bgl*I restriction digestion and hybridization with 7.2 kb rRNA probe of pKK3535 plasmid using DIG DNA labelling and detection kit. Universal VC1 primer was used for rep-PCR.**

***Results*: PFGE generated 15 pulsotypes, of which four matched the published pulsotypes and there were 11 new pulsotypes. PFGE was the most discriminatory method that could differentiate between isolates belonging to single ribotype. Pulsotype P1 corresponding to known pulsotype H1 was the major pulsotype till 2003. Pulsotype P3 corresponding to known pulsotype L emerged in 2004. The 2007 outbreaks in Punjab and Haryana were caused by P5 though P1 and P3 were isolated from the sporadic cases from the same region. The 2008 outbreak was caused by pulsotypes P6 and P7. Ribotype IV was the most predominant followed by RIII. This ribotype was not isolated after 2003 and ribotype IV became the most predominant 2004 onwards. Of the two unknown ribotypes (UNI and UN2), UNI was more common (27 isolates). Rep-PCR was the least discriminatory and divided all clinical isolates into four major profiles. The dendrogram analysis of PFGE revealed similarity of some clinical isolates with environmental isolates indicating the genetic relatedness.**

***Interpretation & conclusion*: Our findings showed that Rep-PCR was least discriminatory method. Ribotyping was a reliable and reproducible method. Ribotype IV was predominant ribotype followed by RIII. A total of 15 pulsotypes were generated and 11 of these were not reported earlier. Genetic relatedness was shown by clinical and environmental isolates which needs to be confirmed in future studies.**

**Key words** Molecular typing - PFGE - rep-PCR - ribotyping - *Vibrio cholerae*

Cholera is an acute diarrhoeal disease endemic in India and whole of the Ganges basin from where it has spread in the form of epidemics and pandemics to several other parts of the world with devastating effects. Recent years have witnessed resurgence in global incidence of cholera cases as reported to WHO. Globally, cholera alone causes 120,000 deaths annually[1]. Cholera is endemic in southern Asia and in several parts of Africa and Latin America where seasonal outbreaks occur[2]. At the tertiary care referral centre at Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, north India, cases of cholera are referred from nearby areas of Punjab, Haryana, Himachal Pradesh, Uttar Pradesh and Uttarakhand. *Vibrio cholerae* serogroup O139 was isolated during a small outbreak of cholera in 1994 in Chandigarh[3]. After this outbreak, cholera has been quiescent with a few sporadic cases. These cases were recorded mostly between July and September. In July 2002, a large outbreak of cholera occurred in Chandigarh. The pockets affected had a mixture of urban slum and rural areas. Spill-over of cholera cases also occurred in the nearby areas of Punjab and Haryana affecting more than a 1000 people[4]. A gross contamination of drinking water occurred because of breakage in sewage pipes. The outbreak was due to *V. cholerae* O1 biotype El Tor serotype Ogawa. In July 2004, another outbreak of cholera occurred in a laborours' encampment in Chandigarh[5]. *V. cholerae* was also isolated from the hand pump, the soil surrounding hand pump and a pond in the affected areas. Similarly, between July and September 2007, six clusters of cholera outbreak were recorded involving four deaths. These outbreaks occurred in Punjab and Haryana with a total of 745 admitted cases of cholera (attack rate, 183 cases per thousand population). Number of cases varied from 15 to 400 in six clusters, affecting mainly adult population[6]. Two clusters of cholera occurred in 2008 (Punjab and Haryana). All outbreaks were identified due to *V. cholerae* O1 Ogawa (unpublished data). Since the genetic characteristics of the *V. cholerae* O1 isolated were not fully explored, it was decided to study the molecular epidemiology of outbreak and sporadic cholera associated isolates from northern region. In the present study isolates were typed by pulsed-field gel electrophoresis (PFGE), ribotyping and rep-PCR to study the clonality. *V. cholerae* isolated from water from the same region were also included.

## Material & Methods

The present study was conducted at the Enteric Laboratory, Department of Medical Microbiology, Postgraduate Institute of Medical Education & Research, Chandigarh, India.

*V. cholerae isolates used for molecular typing*: A total of 173 isolates were included in the molecular typing that were isolated between 2002 and 2008. These included outbreak isolates from July 2002 and July 2004 in Chandigarh. In 2007, six clusters of cholera outbreaks were confirmed in Punjab and Haryana (Ambala, Raili, Mohali, Noorpur, Kurali, Panchkula) and two clusters of cholera occurred in Panchkula (Haryana) and Mohali districts (Punjab) in 2008. In 2003, 2005 and 2006, very few cases of cholera were reported to PGIMER, mainly from neighbouring States. Sporadic isolates from various States were also included (Table I).

*Biochemical, serological characterization*: Isolates of *V. cholerae* were characterized biochemically using standard methods[7]. The isolates were positive for oxidase test, string test, cholera red reaction, lysine and orinithine decarboxyaltion, and confirmed by serotyping using commercially available antisera (Denka-Seiken Co. Ltd., Tokyo, Japan). Biotyping was performed with chick cell agglutination, sheep RBC haemolysis (tube haemolysis with 1% glycerol), polymixin B sensitivity and Voges-Proskauer (VP) test[7]. Isolates were preserved in trypticase soya broth with glycerol at -70 °C.

*Isolation of V. cholerae from water samples*: Water samples (n=236) were collected from cholera affected areas. These included 26 from tube wells, 88 from taps, 53 from hand pumps, 46 from water stored in tanks, buckets and cans, 7 from pond water, 5 from *naalas* (large drains), 10 from river bodies and one from water tanker. Water (250 ml) was collected in the sterile container and passed through a membrane filter with pore size of 0.45µm (Millipore Corporation, USA). After filtration, the membrane was placed in 10 ml alkaline peptone water (APW, *p*H 8.5). After 6 h of incubation a subculture was made from the alkaline peptone water (APW) onto thiosulphate bile salt sucrose agar (TCBS, DIFCO Laboratories, USA). After 24-48 h incubation, typical golden yellow colonies were picked up and subcultured for further identification.

*Pulsed-field gel electrophoresis (PFGE)*: PFGE was performed according to the PulseNet 1-day standardized PFGE protocol for subtyping *V. cholerae* O1 *(http://pulsenetinternational.org)*. Fifty isolates were tested in the PFGE and selection was made based on the year and place of isolation. Briefly, an isolated colony from an individual *V. cholerae* isolate was streaked

**Table I.** Break-up of *V. cholerae* isolates studied

| Year | Place (no. of isolates) | Number |
|---|---|---|
| 2002 outbreak | Chandigarh outbreak (49), sporadic isolate from Ropar (1) | 50 |
| 2003 sporadic | Chandigarh (4), Karnal (2), Pinjore (2), Panipat (1), Panchkula (1) | 10 |
| 2004 outbreak plus sporadic | Chandigarh outbreak (8) plus sporadic cases from Kurukshetra (1), Mohali (4), Ambala (1), Nawashar (2), Lakhnaur Panjab (1) Morinda (1), Ropar (4), Solan (2), Amritsar (1) | 25 |
| 2006 sporadic cases | Ropar (2), Mohali (2), Gorakhpur (1) Chandigarh (3), Rajpura (2) Sangrur (1), Saharanpur (1), Baddi (HP) 1, Fatehgarh (Pb) 1, Ferojpur (1) | 15 |
| 2007 outbreaks (six outbreaks) plus sporadic | Ambala (2), Noorpur (2) Panchkula (2), in Haryana, Balongi (1), Mohali (7), Kurali (3) in Punjab, Sporadic cases from Baddi Himachal Pradesh (1), Chandigarh (6), Panchkula (2), Haridwar (1), Himachal Pradesh (1) | 28 |
| 2008 outbreaks in Panchkula (Haryana) and Mohali (Punjab) | Panchkula (4), Mohali (9), sporadic cases from Chandigarh (2) | 15 |
| 2007 (Sporadic cases from North Delhi) | Delhi | 20 |
| Enviornmental isolates 2007 | From Punjab, Haryana and Chandigarh | 10 |
| Total No. of isolates | | 173 |

Two isolates were submitted from Jammu and Kashmir in 2004, which could not be confirmed as *V. cholerae*, therefore, not included. Haryana - Pinjore, Karnal, Panipat, Kurukshetra, Ambala, Noorpur, Panchkula; Panjab - Mohali, Balongi, Kurali, Nawashahar, Lakhnaur, Morinda, Ropar, Amritsar, Rajpura, Fathegarh, Ferozpur, Sangrur; Himachal Pradesh (HP) - Baddi, Solan; Uttarakhand - Haridwar; Uttar Pradesh (UP) - Saharanpur, Gorakhpur

onto blood agar and incubated at 37°C for 14 to 18 h. Agarose plugs were prepared by mixing equal volumes of bacterial suspensions with molten 1 per cent SeaKem Gold agarose (Cambrex Corporation, USA), 1 per cent N-lauroyl sarcosine (Sigma Chemicals Co., USA) and 0.1 mg/ml Proteinase K (Invitrogen, USA). Cells were lysed for 1 h in water bath at 55°C and washed 2 times with distilled water and 4 times with TE buffer at 50°C before restriction digestion, which was carried out with 50 U of *Not*I (Fermantas International Inc, Canada) at 37°C for overnight. Restriction fragments were separated on a CHEF DR III (Bio-Rad, USA). The total run time was 19 h with 2 blocks, in the 1st block switch time of 2 to 10 sec for 13 h and in 2nd block switch time of 20 to 25 sec for 6 h with a voltage gradient of 6 V per cm. The gel was stained with 1 µl/mg of ethidium bromide solution (Sigma Chemicals Co., USA) and destained with distilled water. Images were captured with a Gel Doc system (BioRad, Hercules, USA).

*Ribotyping*: *V. cholerae* isolates were cultured in Luria-Bertani (L-B) medium, and 3 ml of culture was used to extract and purify the genomic DNA using the Genomic DNA Extraction kit (Fermantas International Inc, Burlington, Ontario Canada). Aliquots of genomic DNA of each isolate was digested with *Bgl*I for ribotyping. The digested DNA fragments were separated by agarose gel electrophoresis (0.7% gel) and were blotted on to nylon membranes. The probe DNA was 7.5 kb *BamH*I fragment of pKK3535[8] which is a pBR322 derived plasmid containing an *Escherichia coli* rRNA operon consisting of one copy of the genes coding for 5S, 16S, and 23 rRNA, and $tRNA^{glu}$. The probe DNA was labelled using DIG DNA labelling kit (Roche Applied Science, USA). For southern hybridization, the membranes were prehybridized at 42°C for 2 h in a solution containing 2x SSC (1× SSC is 0.15 M NaCl + 0.015 M sodium citrate), 1 per cent blocking reagent, 0.1 per cent *N*-lauryl sarcosine, 0.02 per cent sodium dodecyl sulphate (SDS), and 50 per cent formamide. The membranes were then hybridized with the freshly denatured digoxigenin-labelled gene probes at 42°C for 12 h. The hybridized membranes were washed twice in 2x SSC- 0.1 per cent SDS for 5 min at room temperature and then twice in 0.1X SSC-0.1 per cent SDS for 15 min at 68°C. Non radioactive detection was based on digoxigenin- anti-digoxigenin enzyme-linked immunosorbent assay, according to the manufacturer's instructions of the DNA labelling and detection kit.

*Rep-PCR fingerprinting using VC1 primer*: The genomic DNA was isolated by lysis method and purified using phenol-chloroform method[9]. The oligonucleotide primer was obtained from Genxbio Health Science (P) Ltd, Delhi, India. Rep-PCR primer VC1with the sequence 5'-AACTGTGTGATTAGGATCAACGAA-3'was used for the amplification. This primer was designed from ERIC related sequence of *V. cholerae* designed by Sharples and Lloyds[10]. The DNA was amplified using the amplification cycle as 95°C for 5 min to denature template, followed by low stringency cycles of 94°C for 5 min, 40°C for 5 min, 72°C for 5 min and 30 cycles of high stringency of 94°C for 1 min, 55°C for 1 min 72°C for 2 min and final extension of 72°C for 10 min. The amplified products were separated by agrose gel electrophoresis in 1.5 per cent agrose in 0.5x TBE containing ethidium bromide (1 µg/ml) at 60 V for 4-5 h. Gels were photographed using Alpha imager™-3400 (Santa Clara, USA). The fingerprints obtained by Rep-PCR were visually compared. All samples were prepared and examined on at least two different occasions.

## Results

Molecular typing was performed for 163 representative clinical and 10 environmental isolates obtained from 236 water samples. All clinical isolates were phenotypically biotype El Tor. Majority of isolates were Ogawa, only a few were Inaba (17 isolates). All environmental isolates were typed as non O1 non O139. Table II shows the list of isolates used in the PFGE (50 isolates), ribotyping (173) and Rep-PCR (173).

*Analysis of results of PFGE*: The PFGE profile differentiated the 50 *V. cholerae* isolates into 15 types (P1 to P15) according to the Tanover's criteria[11]. Overall, 15 patterns (Figs 1 & 2) of PFGE were obtained with 12 isolates in type P1, 11 in type P3, six in type P5, four in type P6, three in type P12, two each in P7, P4, P11 and P 13 types. Six patterns were unique with overall discriminatory index of 0.838 for PFGE. No association was obtained between the PFGE pattern and the

**Table II.** Number of isolates studied by various typing methods

| Year | PFGE | Rep-PCR, ribotyping |
|---|---|---|
| 2002 | 9 | 50 |
| 2003 | 4 | 10 |
| 2004 | 10 | 25 |
| 2006 | 4 | 15 |
| 2007 | 9 | 28 (+20 from Delhi) |
| 2008 | 9 | 15 |
| Environmental | 5 | 10 |
| Total | 50 | 173 |

PFGE, pulsed-field gel electrophoresis

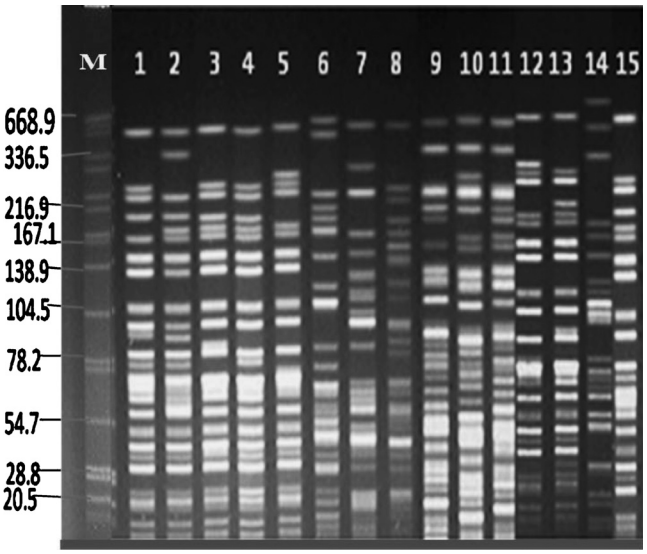


**Fig. 1.** PFGE fragment patterns of Not-I digested total cellular DNA from representative *V. cholerae* isolated in Chandigarh, India. Lane M: Molecular weight marker *Salmonella enterica* serovar Braenderup H9182, Lanes 1-15 are the representative 15 pulsotypes obtained.

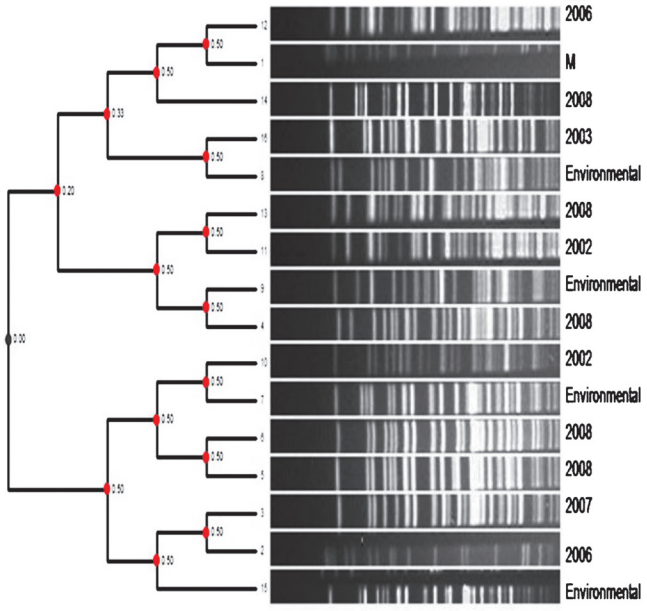


**Fig. 2.** Dendrogram of representative pulsotypes obtained by PFGE analysis. Distance matrix method: Dice coefficient, cluster method-UPGMA. Year represents the year of isolation and all environmental isolates were non agglutinating.

**Table III.** Year-wise analysis of pulsotypes from outbreaks and sporadic cases

| Year | PFGE patterns (No. of isolates) | Outbreaks regions | Sporadic cases |
|---|---|---|---|
| 2002 | P1 (6),P 2(1), P11 (1), P12 (1) | Chandigarh - P1(5), P2 (1), P11(1), P12 (1) | Ropar (Punjab) P1(1) |
| 2003 | P1(4) | - | Karnal, Pinjore, Panipat, Chandigarh (1 each) |
| 2004 | P3 (7), P 4(2), P13 (1) | Chandigarh -P3(6), P4(1), P13(1) | Kurukeshtra - P3(1), Mohali P4(1) |
| 2006 | P3 (1), P11(1), P12(2) | - | Ropar-P3(1), Chandigarh-P11(1), Mohali -P12(1), Gorakhpur- P12(1) |
| 2007 | P1(2), P3(1), P5(6) | Balongi P5(1), P1(1), Noorpur P5(2), Paleora P1(1), Mohali P5(1) | Rajpura P5(1), Mohali P5 (1), Mandi (HP) P3(1) |
| 2008 | P6(4), P3(2), P13(1), P7(2), | Mohali P6(1), P7(1), Panchkula P6(1), P13(1), Balongi P3(2), P7(1) | Badmajra P6(2), |
| Environment 2007 | P8(1), P9(1), P10(1), P14(1), P15(1) | | |

Total types obtained = 15

geographic distribution of isolates and also with year of isolation. Five environmental *V. cholerae* non-O1 non-O139 isolates exhibited unique profiles different from the clinical *V. cholerae* O1 isolates. Four patterns P1, P2, P3 and P11 matched with the known pulsotypes (H1, A2, L and O). Twenty four isolates belonged to 11 unknown pulsotypes including all environmental isolates which exhibited different profiles. The year-wise details of isolates along with different pulsotypes are summarised in Table III. It was interesting to note that even the outbreaks were caused by isolates of multiple pulsotypes, *e.g.* though P1(H1) was the major pulsotype during 2002, P2(A2), P11 (O) and P12 also existed, and from sporadic cases, only pulsotype P1(H1) was obtained. In 2003, only pulsotype P1 was recorded from isolates of Karnal, Pinjore, Panipat, and Chandigarh. In 2004, pulsotype P3(L) became predominant along with P4 though some isolates still belonged to pulsotype P1. In 2006, from sporadic cases pulsotypes 3, 11, 12 were obtained. In 2007, six outbreaks occurred in Punjab and Haryana, mainly with pulsotype P5 though P1 and P3 were isolated from sporadic cases from the same region. In 2008, outbreaks were due to P6, P3 and P7. Environmental isolates were different, though dendogram analysis (Fig. 2) showed matching profile with some clinical isolates. This indicates the clonal relatedness of these two major groups of *V. cholerae*. Though many pulsotypes were circulating in the region only some of them caused outbreaks.

*Ribotyping results*: Among 163 clinical O1 isolates, ribotyping produced four restriction patterns with discriminatory index of 0.48 (Fig.3 and 4). Ribotype IV was the commonest (120 isolates) pattern while 16 isolates belonged to ribotype III. There were two unknown types, marked UNI and UN2. UNI was more common (27 isolates) and the UN2 was seen with environmental isolates. It was observed that during a single outbreak in 2002, which was quite large and due to breakage of sewage pipes, ribotypes III, IV and UNI were found. Ribotype III was not detected after 2003, and ribotype IV was the major type. Similarly, pulsotypes were also different during the same year. Table IV shows year-wise analysis of ribotypes. The 2002 outbreak was caused by multiple ribotypes. The outbreaks of 2004, 2007 and 2008 were caused by ribotype IV. However, the sporadic cases were caused by different ribotypes. During 2002, 2003, 2007 and 2008 cholera outbreaks, more than one ribotypes were disseminated but during 2004 and 2006, there was uniformity in the distribution of ribotypes.

**Table IV.** Year-wise ribotype analysis of the *V. cholerae* O1

| Year of isolation | Ribotypes (No. of isolates) |
|---|---|
| 2002 | RIII(12), R1V(20) UN1(18) |
| 2003 | RIII(4), R1V(6) |
| 2004 | R1V(25) |
| 2006 | R1V(15) |
| 2007 | UN1(6) R1V(42) |
| 2008 | UN1(3) R1V(12) |
| Environmental isolates | UN2(10) |
| Total | 173 |

UN, unknown type

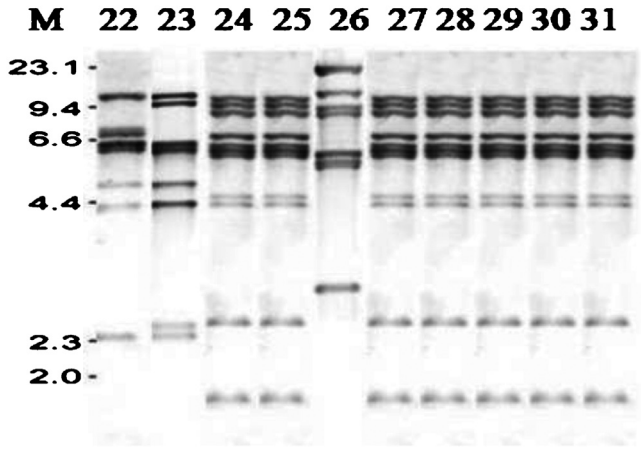


**Fig. 3.** Molecular weight marker, Lane 22, UN1, Lane 23 ribotype III, Lanes 24, 25, 27 through 31 Ribotype IV, Lane 26: Unknown type II.

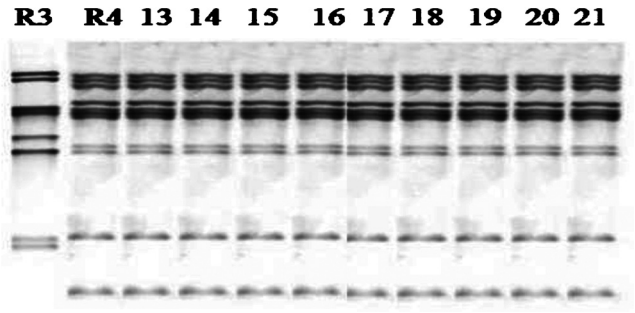


**Fig. 4.** Ribotypes of *V. cholerae* isolates observed after hybridization with digoxigenin-labeled PKK3535 probe. R4: Standard strain from NICED ribotype IV, R3: Standard strain from NICED ribotype III, Lane 13 through 21-Ribotype IV.

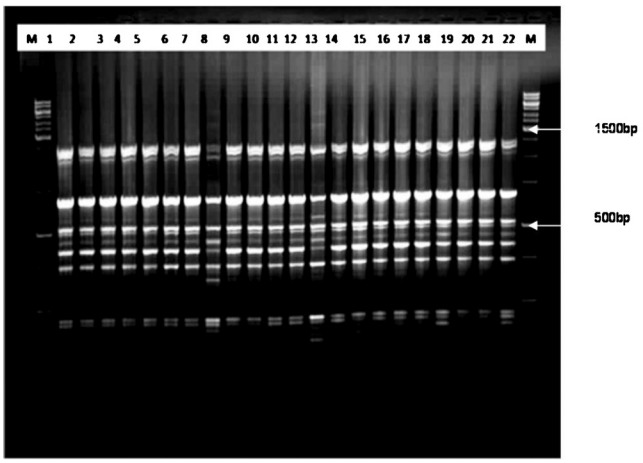


**Fig. 5.** Rep-PCR analysis of *V. cholerae* isolates. M- Molecular weight marker. Lanes- 1-22 are *V. cholerae* collected at different frame of time. Lane M- Molecular marker lane Type 1 (Rp1) 1,2,3, 4,5,6,7,9,10,11,12,14,15,16,17,18,20,21 Type 2 (Rp2) Lane, 19, 22 Type 3 (Rp3) Lane 8 Type 4 (Rp 4) Lane 13.

*Results of Rep-PCR*: Rep-PCR generated four major profiles in clinical isolates (Fig. 5). Rep-PCR differentiated clearly between the pathogenic and non-pathogenic isolates as environmental isolates exhibited distinct profiles. Type Rp1 was the commonest (130 isolates, 80%), followed by Rp2 (17, isolates 10.4%), Rp4 (10 isolates, 6.13%) and Rp3 (6 isolates, 3.68%). Rep-PCR was the least discriminatory method with Simpson's index of diversity coefficient of 0.35. However, except three, most of the environmental isolates gave unique patterns and these profiles did not match with any of clinical isolates. In addition, there was no association between the year and origin of the isolates as the diversity co-efficient was low.

**Table V.** Comparison of isolates typed by all three methods (n=50)

| Year of isolation (No.) | Ribotypes | Pulsotypes | Rep-PCR types |
|---|---|---|---|
| 2002 (9) | RIII(3), R1V(4) UN1(2) | P1 (6), P 2(1), 11 (1), 12 (1) | Rp1 (7), Rp4 (2) |
| 2003 (4) | RIII(2), R1V(2) | P1(4) | Rp1 (2), Rp3(2) |
| 2004 (10) | R1V(10) | P3 (7), P4(2), P13 (1) | Rp1 (7), Rp2 (1), Rp 3(1), Rp4(1) |
| 2006 (4) | R1V(4) | P3 (1), P11(1), P12(2) | Rp1 (4) |
| 2007 (9) | UN1(3) R1V(6) | P1(2), P3(1), P5(6) | Rp1 (7), Rp2(2) |
| 2008 (9) | UN1(4) R1V(5) | P6(4), P3(2), P13(1), P7(2), | Rp1 (7), Rp4 (2) |
| Environment 2007 (5) | UN2(5) | P8(1), P9(1), P10(1), P14(1), P15(1) | Rp5(2), 3 isolates gave variable patterns Rp6-Rp8(1 each) |
| Total | 50 | 50 | 50 |

Table V shows the comparison between three typing methods for the 50 isolates which were typed by all three methods. PFGE easily distinguished among isolates exhibiting same ribotype and repPCR patterns. Multiple clones of *V. cholerae* O1 existed at any given time. The year-wise analysis of isolates collected from different regions indicated presence of different clones associated with outbreaks and sporadic infection of cholera. Each outbreak was caused by more than one pulsotype.

## Discussion

Cholera is an acute diarrhoeal disease endemic in India causing seasonal outbreaks. Recent years have witnessed resurgence of cholera in Chandigarh and several north western States mainly during 2002-2008. The outbreaks were due to contaminated drinking water and occurred in semi urban and rural populations. All the outbreaks were caused by *V. cholerae* O1 El Tor biotype. The genetic diversity or clonality among *V. cholerae* causing outbreak and sporadic cholera was studied in order to ascertain the circulation of pulsotypes and ribotypes and genetic similarity between the clinical and environmental isolates, if any. A total of 163 clinical and 10 environmental isolates were analyzed. Forty five *V. cholerae* O1 isolates representing all the outbreaks as well as sporadic cases and five from environment were subjected to PFGE. Ribotyping and Rep-PCR were performed for all the isolates. Ribotype IV was the most predominant followed by RIII. RIII was not isolated after 2003 and ribotype IV became the most predominant from 2004 onwards. Study by Sharma *et al*[12] showed that RIII emerged in 1993 after the spread of O139 serogroup in many cholera endemic regions. Ribotype IV appeared for the first time during 2004-2005[13]. In 2004, we reported the serotype switch from Ogawa to Inaba and in many northern parts of the country[14]. The re-emerged Inaba isolates from Delhi, Tripura and Gwalior were found to belong to this new ribotype IV[14]. Our findings showed that the ribotype RIV existed in northern region during 2002 among Ogawa isolates also. Further comparison of the ribotypes revealed that during 2002, 2003, 2007 and 2008 more than one ribotypes were identified among *V. cholerae* O1, while during 2004 and 2006, uniform ribotype pattern was observed. Ribotyping which uses labelled *E. coli* rRNA to probe the ubiquitous and polymorphic rDNA loci is widely accepted as a powerful epidemiological tool. Popovic *et al*[15] proposed a standardized scheme and showed that strains causing the seventh cholera pandemic belonged to several ribotypes. Ribotyping as observed by earlier investigators is a straight forward approach to study the clonal structure of bacterial populations and is highly reproducible. Our study also confirms the high degree of reproducibility of ribotyping as previously reported[16].

PFGE generated 15 pulsotypes, of which four matched with the published pulsotypes. Some of the isolates were found to be unique to our region with hitherto unreported pulsotypes, which need further characterization. PFGE was the most discriminatory method and could differentiate between isolates belonging to single ribotype. P1 corresponding to H1 was the major pulsotype till 2003. Dutta *et al*[13] have also shown that H and H1 were the dominant pulsotypes as seen in our study. P3 corresponding to pulsotype L emerged in 2004, as also shown by Dutta *et al*[13]. The 2007 cholera outbreaks in Punjab and Haryana were caused by isolates mainly belonging to pulsotypes P5 though P1 and P3 were associated with sporadic cases from the same region. The 2008 outbreak was caused by pulsotypes P6, P13 and P7. The dendrogram analysis of PFGE revealed similarity of some clinical isolates with environmental isolates indicating the genetic relatedness. Further study with the inclusion of large number of environmental and clinical isolates is needed to confirm the present findings. A detailed study on environmental reservoir of *V. cholerae* may shed more light on preventing such outbreaks in future.

Rep-PCR was the least discriminatory and separated all clinical isolates into four major profiles. However, it was a good technique to differentiate clinical toxigenic isolates from environmental non toxigenic isolates.

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