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Genomic characterization of multidrug-resistant *Vibrio Cholerae* O1 El Tor strains across diverse Indian geographic regions

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Abstract

Cholera poses a major health risk in several countries worldwide, even in the present century. Hence, it is very important to develop new therapeutic and diagnostic methods for restricting the transmission of the disease. Continued research in the field is necessary for understanding how the dynamics of bacterial virulence, pathogenesis and survival strategies keep changing. In the present study, a total of 20 clinical Vibrio cholerae O1 four strains from each year, from all over the country, were selected from different parts of India were investigated to analyze their genotypic and phenotypic dynamicity. The strains studied mostly used Ogawa and T-27 as the prevalent phage type. The study revealed an alarming situation of the emergence of Multidrug resistance. These strains from India emerged in the form of Haitian ctxB and Haitian tcpA. This is quiet alarming to note the shifting of genotype in V. cholerae strains in India. PFGE analysis showed that overall PFGE similarity between the strain about 80% with fourteen different patterns. Additionally, rabbit ileal loop assay showed the toxicity of V. cholerae strains. Overall, the study showed the emergence of multidrug resistance V.choleraehybrid strain circulating throughout the country.

Keywords: *Vibrio Cholerae*, El Tor, Phage typing, Multidrug resistance, Rabbit ileal loop assay.

Introduction

Gram-negative bacteria *Vibrio Cholerae* O1 is the causative agent for the epidemic and endemic cholera throughout the globe, particularly in developing countries. The disease is considerable health burden due to its poor hygienic condition. The clinical O1 strains can further be segmented into two serotypes Ogawa and Inaba. *V. cholerae* O1 is bifurcated into two biotypes as classical and El Tor, which differ biochemically and in levels of virulency. Serologically, *V. Cholerae* are subdivided in more than 200 "O" serogroups, where O1 and O139 serogroup are mainly causative agents for cholera epidemics[1]. It is well - known cholera has been endemic in the Gangetic Delta region of eastern India for centuries, most of the countries of sub-Saharan Africa and Latin America affected severely during the 7th pandemic and later are considered areas of endemicity[2].

Cholera toxin (CT), which is encoded by the *ctx*AB genes located in the CTX̃ prophage, is the key virulence factor that is directly control for the development of diarrhoea throughout the infection[3]. Currently it is shown that, *V. Cholerae* O1 biotype El Tor includes the following variants: the Matlab variants, combined of both the classical and El Tor biotypes; the Mozambique variants, which contain a typical El Tor genome including a tandem repeat in the classical CTX prophage of small chromosome; and, finally, the altered El Tor variants, which have a distinctive El Tor biotype with CTX prophage responsible for producing the classical cholera toxin[4]. We previously included strains of *V. cholerae* isolated between 1961 and 2010. It has been shown that the changing scenario of pattern for resistance and clonal relationship within strains worldwide in the past fifty years[5]. It is reported that the major phage type was T-27 followed by phage type T-26, T-22, T-19, T-13 and T-7. Based on the outcome mismatch amplification mutation assay PCR (MAMA-PCR) demonstrated that the *ctx*B El Tor genotype predominated. The seventh-pandemic El Tor prototype strains were supplanted by classical *ctx*B in El Tor biotype strains throughout the majority of cholera-endemic regions, according to studies conducted in Asia and Africa. The fast spread of resistance over the last 30 years and the need for appropriate treatment to safeguard vulnerable populations are illustrated by the regional resistance of epidemic clones in India. Strong evidence of the evolving cholera historical situation regarding the pattern of resistance and clonal relationships among strains globally was also established by this study[6]. The preceding study is being continued in this one.

Materials and Methods

Bacterial strains: The strains of *V. cholerae* used in this study were collected from our in-house strain library of epidemic and sporadic *V. cholerae* isolates received from diverse parts of the country. A total of 20 strains from nine states in India were included in this study. Strains were confirmed by standard methodology in our laboratory[7].

Serology was performed using polyvalent, anti-Ogawa and anti-Inaba antisera (Denka Seiken Co. Ltd., Tokyo, Japan). A total of 18 out of 20 strains were incorporated for Polymyxin B susceptibility (50U) test[8].

Biotype differentiation (Polymyxin B, Gr. IV and El Tor Gr. V phage): All these strains phenotypically differentiated with Polymyxin B (50 U ml^{-1}), luria broth (LB) plate susceptibility assay, Gr. IV phage and El Tor Gr. V phage sensitivity assay. A single colony from LB plate was inoculated into LB media for 4–6 h. One loopful of this culture was streaked on Luria agar (LA) plate with polymyxin B (50 U ml^{-1}) and incubated overnight at 37°C. A corresponding set of plates without polymyxin B were prepared to check whether the sensitive strains were viable and still capable of growing on LA plate. Additionally, Gr. IV phage and El Tor Gr. V phage sensitivity assay were performed on Nutrient broth (NB) and Nutrient agar (NA) plate. Phage lysate was dropped onto the growth of the bacteria. After incubation, each reaction was recorded as positive showing the appearance of clearing zone or lysis[8].

Phage typing: All strains of *V. cholerae* isolated from different endemic parts of the country were included in this study. These strains were received for confirmation, biotyping, serotyping and phage typing study at NICED. This was performed by the standard methodology routinely used at the Vibrio Phage Reference Laboratory, NICED[9]. *V.cholerae* MAK 757 (ATCC 51352), the propagating strain was used as a control for O1 strains.

Antibiogram: Study all *V. cholerae* O1 strains were tested for antimicrobial susceptibility by the method of Kirby Bauer using antibiotic disks (BD Difco Laboratories, Sparks, MD) containing ampicillin (10µg), azithromycin (15µg), ceftriaxone (30µg), erythromycin (15µg), norfloxacin (10µg), ofloxacin (5µg), trimethoprim/sulfamethoxazole (SXT) (1.25/23.75µg), ciprofloxacin (5µg), Nalidixic acid (15µg), tetracyclin (10µg) and streptomycin (5µg). *Escherichia coli* ATCC 25922 was used as a control strain in each assay[10].

Pulsed-field gel electrophoresis (PFGE): Pulse-field gel electrophoresis (PFGE) was performed following the Pulse-Net standardized PFGE protocol for *V.cholerae*[11]. A representative of 18 out of 20 strains of *V.cholerae*O1 from different years was analyzed for the PFGE. The agarose embedded plugs of *V. cholerae* DNA were digested with 50 Units/µl of NotI (NEB). Salmonella Braenderup H9812 was digested using the molecular size marker XbaI (15 Units/µl), and the DNA fragments were separated electrophoretically on a 1% PFGE grade agarose gel in 0.5xTBE (44.5 mMTris/HCl, 44.5 mM boric acid, 1.0 mM EDTA, pH 8.0) at 14°C using a CHEF DRIII system (Bio-Rad). After electrophoresis, the gels were destained in ElixMilliQ water for 15 minutes, stained for 30 minutes with 1.0 µg ethidium bromide, and captured on camera using the Gel Doc 2000 gel documentation system (Bio-Rad) under UV light. A program called Bionumeric (Applied Maths, Belgium) was used to analyse DNA fingerprint patterns.

Polymerase chain reaction (PCR) assays: Based on the concentration on nucleotide position 203 of the *ctx*B gene, the MAMA-PCR was used to identify sequence polymorphism between the classical and El Tor *ctx*B genes (ctxBCL and ctxBET, respectively). After the electrophoresed onto a 1% agarose gel, amplified products were stained with ethidium bromide (Sigma, St. Louis, MO), and a gel documentation system (Bio-Rad, Hercules, CA) was used to digitally capture the images. Toxin corregulated pilus (*tcp*A), located between positions 617 and 482 bp, is the second pathogenic component for both classical and El Tor *V. cholerae*. The primer used in PCR was listed in Table-2. Agarose gel (1%) documentation was done with ethidium bromide (Sigma, St Louis, MO) staining. Emerald Ready Mix (Takara) was added to each sample in a 12.5µl PCR tube. The reaction mixture of 25µl volume containing 1µl each of forward and reverse primer and 2µl DNA template was added in each of the PCR tube. The Haitian-PCR was employed to detect Haitian type of *ctx*B and *tcp*A of new El Tor variant of *V. Cholerae*[13]. Amplified products were electrophoresed in 1.5% agarose gel and stained with ethidium bromide (Sigma, St Louis, MO). The images were recorded digitally using gel documentation.

Table-1: Primer used in this experiment.

Primer	Primer Sequences (5′→3′)	Product Size (bp)
ctxB-F	ACTATCTTCAGC ATATGCACATGG	200
ctxB–R Classical	CCTGGTACTTCT ACTTGAAACG	190
ctxB-R El Tor	ACAAGTTCATC TTCATGGTCC	191
*tcpA*Classical Fow	CACGATAAGAA AACCGGTCAAGAG	617
*tcpA*Classical Rev	ACCAAATGCAAC GCCGAATGGAGC	
*tcpA*El Tor Fow	GAAGAAGTTTGT AAAAGAAGAACAC	471
*tcpA*Classical Rev	GAAAGGACCCT TCTTTCACGTTG	
*ctxB*Haitian Fow Rev	GTTTTACTATCT TCAGCATATGCGA CCTGGTACTTCTACTTGAAACG	191
*TcpA*Haitian Fow Rev	CCAGCTACCGC AAACGCAGG CCGACTGTAATTGCGAATGC	167

Animal experiments: Ethical Statement: Animals were conducted by following the standard procedure as outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA), Ministry of Environment

and Forest, Government of India. The institutional ethical committee approved the animal experiments at National Institute of Cholera and Enteric Disease. The licensed No. is (Animal House Establishment # 68/GO/ReBi/S/1999/CPCSEA Valid to 17th July 2025; Licence # PRO/117/June2016-June 2019).

Bacterial growth: All the *V. cholerae* strains were grown on TSB (Tryptic soya broth) medium and harvested in phosphate buffer solution (PBS). OD value is OD_{600}=1. Bacterial suspension was introduced (1×10^9 CFU/ml) as inoculum size.

Models for the assessment of toxicity: Rabbit Ileal Loop Assay: To evaluate the toxigenicity of the strains involved, rabbit ileal loop studies were carried out as previously described. Prior to surgery, white male rabbits weighing 1.5–2 kg were fed only water and labium and fasted for 48 hours. Ketamine was administered intramuscularly to the rabbits to induce anaesthesia. Following a laparotomy, the ileum was cleaned and tied into separate, roughly 10-cm loops. 10^9 CFU of the test strain of *V. cholerae* were added to each loop in phosphate-buffered saline (PBS). PBS served as the negative control in this instance. After suturing the intestine back into the peritoneum, the animals were put back in their cages. Rabbits were killed by intravenous pentobarbital injection (150 mg kg−1) after 18 hours[16].

Results and Discussion

Serotypes and biotypes of *V.cholerae* O1 isolates: A total of 20 *V. cholerae* strains serogroup and serotypes confirmed by standard methodology using polyvalent, anti-Ogawa and anti-Inaba antisera (Denka SeikenCo. Ltd., Tokyo,Japan). All the isolates were belonged to *V. cholerae* serogroup O1. Ogawa (92%) Inaba (8%) serotype observed among 20 isolates. These strains were tested with classical Gr. IV phage, El Tor phages (ATCC: B-1 to B-10 ϕ) and also polymyxin B sensitivity assay confirmed as biotype El Tor.

Biotype differentiation: The results were verified with a polymyxin B (50 Uml^{-1}) susceptibility assay. All the strains showing a zone diameter of ≥12mm were found to be growth deficient on plates containing 50Uml^{-1} polymyxin B, with the classical reference strain O395. N16961 was used as control for the resistance phenotype that showed growth as a lawn. A total of 101 strains of *V. cholerae* tested showed resistance to polymyxin B, confirmed as El Tor biotype. These strains were uniformly resistant with Gr. IV phage ϕ and sensitive with El Tor V phage ϕ.

Phage typing: All *V. cholerae* strains were also included in phage typing study (Table-1). Both the new and conventional phage typing scheme of Basu & Mukherjee was incorporated in this study. The most prevalent phage type was T-27 (new phage typing scheme, NP) and T-4 (Basu and Mukherjee typing, BM). The most predominant phage type was encountered as T-27 (76%) followed by T-26 (8.0%), T-22 (4.0%), T-19 (4.0%), T-13 (2.0%), T-7 (2.0%), respectively.

Antibiogram: Among 20 *V. cholerae* O1 strains showed discrete antibiogram patterns (Figure-1). It is showed that 100% of the strains were resistant to ampicillin, 78% to streptomycin, 2% to ciprofloxacin, 88% to SXT, 6% to norfloxacin and 8% to erythromycin, whereas most of the strains were found sensitive to ceftriaxone and ofloxacin. Decade-wise data analysis exposed that in India, *V. cholerae* showing resistance to five drugs (ampicillin, nalidixic acid, streptomycin, tetracyclin and cotrimoxazole) among strains isolated from different parts of country.

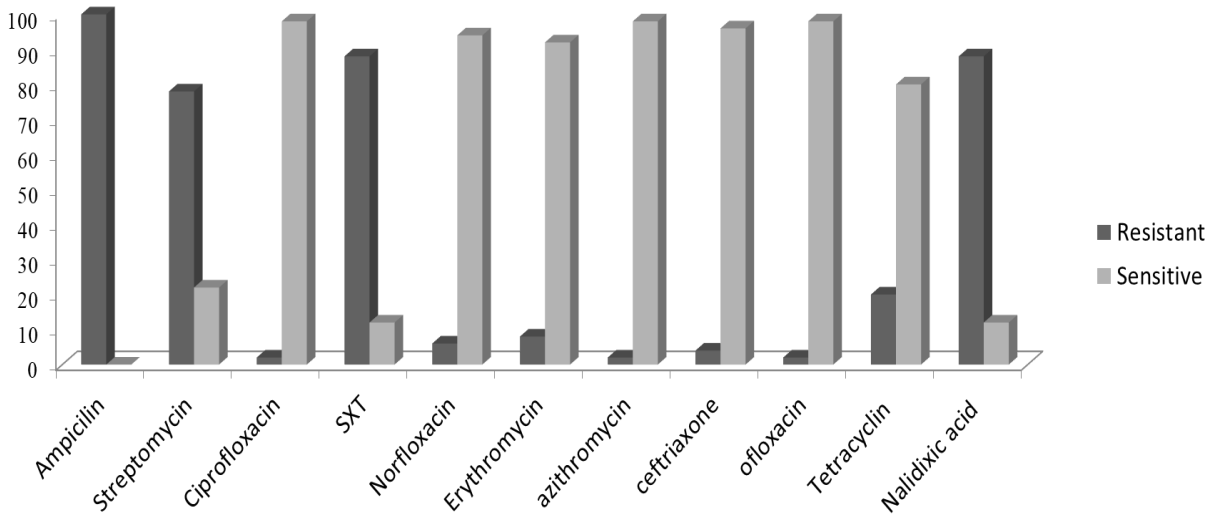

Figure-1: Antibiogram pattern of *V.cholerae* showed multidrug resistant patternampicillin, SXT, nalidixic acid and streptomycin.

Pulsed-field gel electrophoresis (PFGE): PFGE analysis of 18 out of 20 representatives *V. cholerae* strains from different years showed that most of strains have the clonal similarity of more than 90% related with overall pulsed-field gel electrophoresis (Figure-2). Dendogram analysis using Bionumeric software (Applied Maths, Belgium) showed that fourteen different patterns were obtained. Overall the isolates were identified as closely.

Polymerase chain reaction (PCR) assays: The strains were confirmed by MAMA PCR for the presence of *ctxB*^{CL}and *ctxB*^{ET}. A total of 8 (40%) out of 20 strains, denotes as the hybrid strains for the presence of both Classical and El Tor gene (Figure-3). Remaining 9 (40%) out of 20 strains showed the presence of *ctxB*^{CL}classical which are designated as variant. It has been observed that CT negative strain was found in very low number. In this span of time, it has been shown that higher occurrence of *V. cholerae* variant strains (55%) in comparison to hybrid (45%). *V. cholerae* strains were confirmed by PCR for detection of *tcp*A gene for both classical and El Tor genotype. All the *V. cholerae* O1 El Tor strains contain El Tor type of *tcpA*(482 bp). On the contrary, no classical genotype was found in this study. 569B and N16961 were chosen as *V. cholerae* control strain for classical and El Tor respectively. The strains were confirmed by Haitian PCR for the presence of *ctx*Band *tcp*AEl Tor (Table-2). This study reveals, that 40% strains of *V. cholerae* were *ctx*B positive and 66% were *tcp*A positive. Also, 36 out of 50 (72%) strains were positive for both *ctx*B and *tcp*A.

Rabbit ileal Loop Assay: Two of the isolates PL-67 and PL-73 (representing high cytotoxicity) and four representative isolates PL-119, PL-127, PL-133 and PL-156 (representing high haemolytic activity) were examined in rabbit ileal loop assays to measure their fluid accumulation causing potential. PBS and *V. cholerae* O1 El Tor N16961 were chosen as control. It is found that, PL-73, PL-127, PL-156, PL-167, PL-362, PL-189, PL-488 caused fluid accumulation in the range of 0.72-1.25 whereas PL-119, PL-133, PL-157, PL-164, PL-202, PL-230, PL-3014, PL-248 produced the range of 0.4-0.68 ml/cm. The control PBS and N16961 showed 0.4-2.2 and 2.6-5.7 ml/cm respectively.

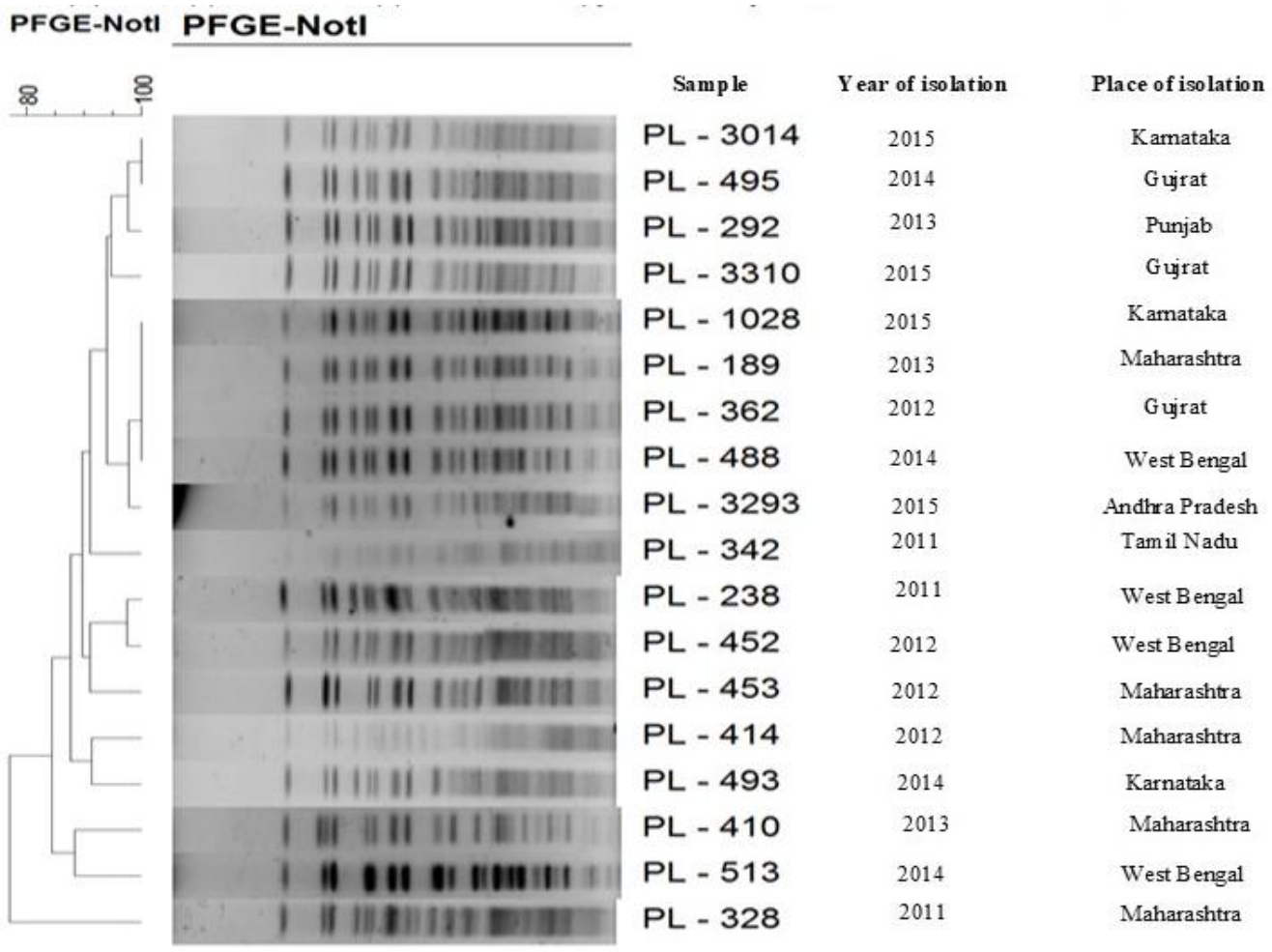

Figure-2: PFGE gel showing four distinct Noti digestion profiles among representative strains isolated from different parts of India.

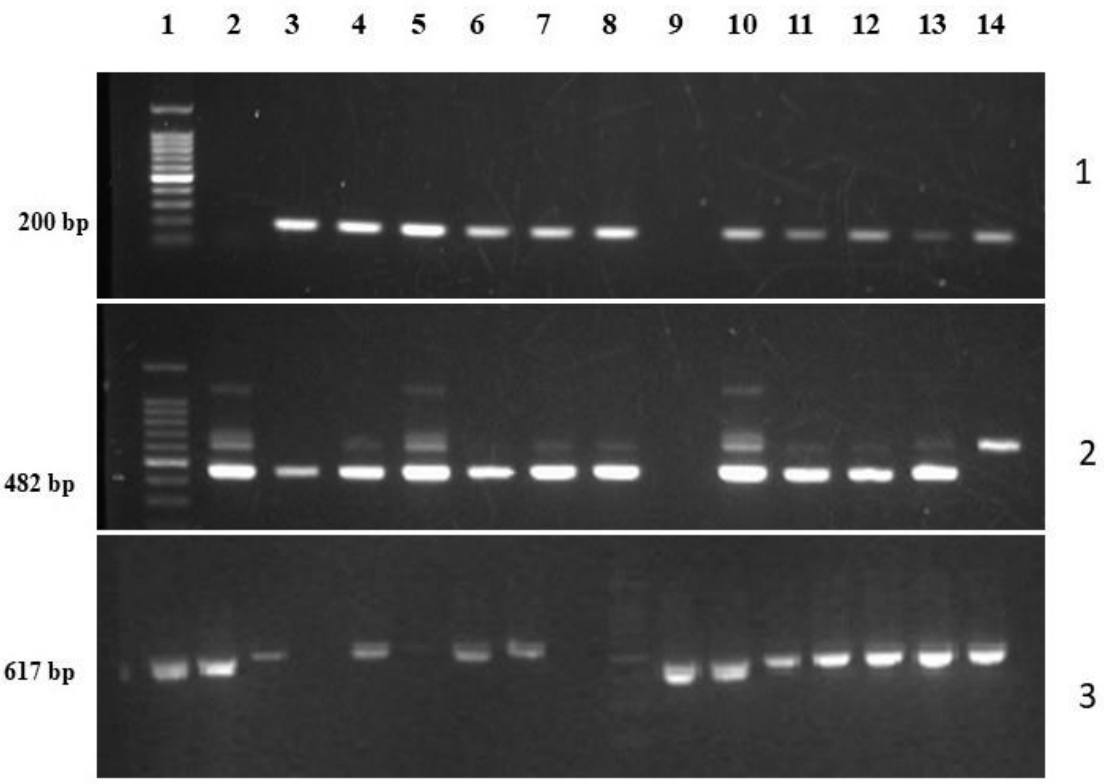

Figure-3: PCR-based assay for *ctx*B, *tcp*A and Haitian *ctx*B, *tcp*A alleles in *V. cholerae* O1 isolates from different states in India. MAMA-PCR was performed for representative *V. cholerae* O1 strains collected from different states in India to detect the type of *ctx*B allele present using primers (ctxB F3/ctxBRv-Cla) for El Tor variant *ctx*B (1) to detect the type of *tcp*A allele present using primers (*tcp*AF/*tcp*A El-Rev) for El Tor variant (2) and (*tcp*AF1/*tcp*A El-Rev) for El Tor *tcp*A (b2); for Haitian *ctx*B and *tcp*A (3). O395 (classical) and N16961 (El Tor) for 1,2,3. The left lane (lane 1) contains a 100-bp ladder for (1) and (2), Lane 8 for (3).

Figure-4: Illustration of the ileal loop experiment used to measure enterotoxigenic activity in rabbits. Illustration of the ileal loop in rabbits of various clinical strains. Examination of a small number of representative *V. cholerae* strains' fluid buildup. Each strain was implanted with 10^9 CFU/mL in rabbit ileal loops. N16961 (O1 El Tor), (PL-119, PL-127, PL-133 and PL-156) El Tor Ogawa in PBS and incubated for 18 h.

Table-2: PCR data of *ctx*B (classical, El Tor) and *tcp*A (classical, El Tor) of *V.cholerae* O1 strains.

Serial No	Name of Strain	State of Isolation	Biotype	Serotype	Phage type	PCR Assay				Ileal loop (FA ratio)
						ctxB^{CL}	*ctxB*^{ET}	*tcp*A^{CL}	*tcp*A^{ET}	
1.	Pl- 41	Maharashtra	El Tor	Ogawa	27	+	-	-	+	0.71
2.	PL-54	Tamil Nadu	El Tor	Ogawa	27	+	-	-	+	0.62
3.	PL-60	West Bengal	El Tor	Ogawa	27	-	-	-	+	0.05
4.	PL-67	Rajasthan	El Tor	Ogawa	27	+	-	-	+	0.07
5.	PL-73	Maharashtra	El Tor	Ogawa	27	+	+	-	+	0.28
6.	PL-119	Gujrat	El Tor	Ogawa	27	+	-	-	+	0.06
7.	PL-127	West Bengal	El Tor	Ogawa	27	+	-	-	+	0.5
8.	PL-133	Maharashtra	El Tor	Ogawa	27	+	-	-	+	1.83
9.	PL-156	Maharashtra	El Tor	Ogawa	27	-	-	-	+	0.62
10.	PL-157	Punjab	El Tor	Ogawa	27	+	-	-	+	0.71
11.	PL-164	Maharashtra	El Tor	Ogawa	27	+	-	-	+	0.57
12.	PL-167	Gujrat	El Tor	Ogawa	27	+	-	-	+	1.25
13.	PL-202	West Bengal	El Tor	Ogawa	13	+	+	-	+	0.55
14.	PL-230	Karnataka	El Tor	Ogawa	27	+	+	-	+	0.54
15.	PL-226	Gujrat	El Tor	Ogawa	27	+	+	-	+	0.76
16.	PL-248	West Bengal	El Tor	Ogawa	27	+	+	-	+	0.42
17.	PL-362	Gujrat	El Tor	Ogawa	7	+	+	-	+	0.5
18.	PL-189	Karnataka	El Tor	Ogawa	27	+	+	-	+	0.68
19.	PL-488	Andhra Pradesh	El Tor	Ogawa	27	+	+	-	+	0.46
20.	PL-3014	Karnataka	El Tor	Ogawa	27	+	+	-	+	1

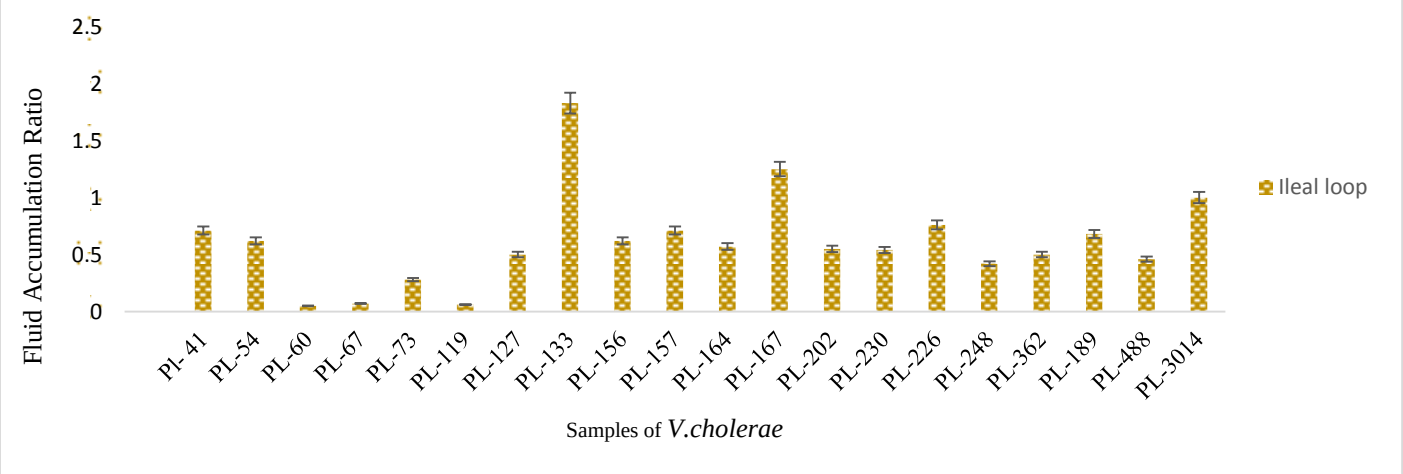

Figure-5: Results of the representative strains are expressed as fluid accumulation (FA) (in millilitres) per loop length (in centimetres).

Discussion: The current situation of the evolving phenotype in relation to the continually malleable genotype of *V. cholerae*, if any, in recent years is the focus of this investigation. The study's findings showed that *V. cholerae* O1 strains are present throughout India and can potentially pose a hazard to public health by causing disaster cholera. According to our findings, the *V. cholerae* O1 isolates linked to cholera nationwide were toxic. Only 8% of them were Inaba serotype strains, whereas the rest (92%) were Ogawa strains. This indicates that Ogawa was a common serotype in India as well as other cholera endemic regions worldwide El Tor is strained[14,15].

All the *V. cholerae* O1 El Tor strains contain El Tor type of *tcpA*, conversely, no classical genotype was found in this study. The PCR assay using two allele-specific forward primers and one common *ctx*B reverse primer for discriminating the classical, El Tor and Haitian type *ctx*B alleles. Randomly, the *V. cholerae* O1 strains carrying both the phenotypes of classical and El Tor biotypes [classical group IV and El Tor ϕ, polymyxin B (50 units) susceptibility and positive for chicken erythrocyte agglutination (CCA) and Voges-Proskauer (VP) test] imply hybrid biotype instead of being typical El Tor.

Phage typing is an ongoing process at this institute since decades. The system is employed as a highly discriminatory technique for biotyping, serotyping and discrimination of strains for epidemiological importance. Here, we found that T-27 was the predominant phage type and distributed to all parts of the country. Diverse phage typing patterns have evolved such as T-7, T-13, T-19, T-22, T-26 respectively, with respect to the new phage typing scheme. T-4 has been growing as the prevalent typing pattern compared with T-2 with typing scheme; the altering typing arrays signify changing resistance patterns towards totally divergent phages[8].

During the last five years period, some new phage types of the new phage typing schemes appeared. These are T-7 (4%), T-19 (2%), T-22 (2%). These types indicating the changing scenario of phage types evolving over the years what we encountered in this study. However, it has been observed that T-27 was predominant phage type as before[16]. Being the predominant phage type, probably, one single clone (T-27) of *V. cholerae* is circulating throughout the country.

The trend for the increase in resistance to ampicillin of *V. cholerae* isolates studied earlier in our laboratory between 1961 to 2010, has been published[17]. This study is supporting our earlier findings of increasing trend of ampicillin resistance. The current study has shown that almost all strains tested were resistant to ampicillin. Antimicrobial susceptibility patterns plays a key role as a phenotypic marker used in the epidemiology in cholera. In this study, *V. cholerae* O1 isolates were resistant to ampicillin, SXT, nalidixic acid, streptomycin and tetracycline. During the course of the study 38% strains of *V. cholerae* were resistance to tetracycline.

Tetracycline resistant strains were also noticed by Barati et al.[17] and Sarkar et al.[18]. Moreover, this study is particularly important and a warning towards the use of ampicillin for the treatment of cholera cases in India. Results showed that all strains were resistant to either one or two antibiotics and hence not a single strain of *V. cholerae* O1 was sensitive to 11 antibiotics used in this study. This study explored MDR *V. cholerae* in every year from 2011 to 2015. In this study, maximum numbers of resistant strains were shown against ampicillin, SXT, nalidixic acid, tetracycline and streptomycin which is also corroborated by other researchers also[21]. Ofloxacin can be considered as a drug of choice. In view of the high level of SXT resistance and contradiction for use of tetracycline and floroquinolones other options need to be considered for the treatment of children.

A trend in multidrug resistance was observed over the years and this trend reported globally[22]. However, the drug resistance power to ciprofloxacin is very low. Multidrug resistant (MDR) bacteria are the problem spreaded worldwide and are a problem for all researchers. Observing the continued therapeutic effectiveness, it is recommended that the drug should be used judiciously for the management of acute diarrheal diseases in order to delay the development of resistance to the drug. The changing trend of antibiotic resistance over time will reflect the treatment regime for *V. cholerae* in the community.

The results of this study showed as a new horizon evidence to formulate new guidelines for the treatment of cholera. Fluid accumulation ratio does not depend on the colonization factor, *tcp*A. Both high and low F/A ratio can be seen in El Tor *tcpA*$^+$ strains, namely, PL-60, PL-67 and PL-362, PL-189, PL-488. This data suggests that, fluid accumulation in rabbit ileal loop does not depend on *tcp*A. On the other hand, it does depend on the status of *ctxB* classical and El Tor. High F/A ratio was evident in cases where hybrid *ctxB* was present. In cases where classical *ctxB* is present, lower F/A ratio was seen. This result suggests the dependence of the status of *ctxB* upon toxicity *in vivo*. In comparison to phage typing, we found that PFGE demonstrated superior epidemiological analysis[24].

A single biotype, serotype, phage type, and antibiotic pattern can be differentiated into several pulsotypes using PFGE. Compared to phage typing, PFGE demonstrated superior type ability and concordance with various geographic locations. For PFGE analysis, we have used 18 of the 20 strains of *V. cholerae* in this investigation.

Conclusion

This study revealed a clonal link between *V. cholerae* O1 strains from several regions of India during its seventh pandemic. However, ongoing surveillance and appropriate monitoring are required to identify minute genetic alterations in the genomes and their consequences for the epidemiology, pathophysiology, and persistence of *V. cholerae* O1.

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