Characterization of *V. cholerae* O1 biotype El Tor serotype Ogawa possessing the *ctxB* gene of the classical biotype isolated from well water associated with the cholera outbreak in Kerala, South India

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ABSTRACT

We investigated 22 water samples (17 well water and five pipe water – both chlorinated) and six soil samples from the surroundings of wells of the households of suspected patients from Palakkad district, Kerala (India), from where a cholera outbreak was reported during June–July 2016. A total of 25 *Vibrio cholerae* isolates were collected from three well water samples during a recent cholera outbreak. Biochemical and serological studies revealed that all of the isolates belonged to serogroup O1, biotype El Tor, serotype Ogawa. PCR assays confirmed the occurrence of *ctxB*, *ctxA, hlyA, tcpA* El Tor,*VPI, ace, zot, ompW, rfb*O1 and *toxR* genes in all isolates. The presence of the *ctxB* gene of the classical biotype in all of the El Tor isolates suggests that it is a new variant of El Tor biotype. Antibiogram profile of all *V. cholerae* O1 isolates revealed resistance towards five classes of antibiotics island and indicates that they were multidrug resistant. ERIC-PCR and PFGE finger prints showed the clonal relationship among the *V. cholerae* O1 isolates. The results of this study revealed the emergence of a new variant of El Tor biotype in the water samples from Palakkad district, from where a cholera outbreak was reported.

Key words | ERIC-PCR, multidrug resistance, PFGE, toxigenic strains, *Vibrio cholerae*, well water

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HIGHLIGHTS

- Presence of altered *V. cholerae* O1 El Tor Ogawa strains in chlorinated well water might constitute a threat to public well-being.
- Isolated strains with virulent genes have potential to become pathogenic.
- Multidrug resistant strains have shown antibiotic resistance towards commonly used antibiotics.
- This research will be useful in ecological and epidemiological studies.

INTRODUCTION

Cholera is an acute gastrointestinal illness characterized by severe watery diarrhoea and is caused by the bacterium *Vibrio cholerae* that is acquired by the ingestion of contaminated food or water. Globally, cholera alone causes 120,000 deaths annually (Sack *et al.* 2006). The Indian sub-continent has been an epicentre for cholera. However, cholera cases are hugely under-reported mainly because disease surveillance is limited. The incidence of cholera is estimated to be 1.6 cases/1,000 population per year or 40/1,000 cases of acute diarrhoea in India (Sharma *et al.* 2017). According to

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the World Health Organization (WHO), a cumulative total of 838,315 cases were notified in India for the time period of 2004 to 2008, as compared to 676,651 cases in the years 2000 to 2004. This represents an approximate 24% rise in the number of cholera cases identified (Kanugo *et al.* 2010). During a ten-year period (1997–2006) study in India, outbreaks were reported in Kerala in multiple years and the states having the highest number of reported outbreaks were West Bengal, Orissa, Maharashtra and Kerala (Kanugo *et al.* 2010). During 2010–2015, cholera was reported in three districts in Kerala during at least three out of five consecutive years, and the state was defined as a cholera endemic state (Ali *et al.* 2017).

The re-emergence of the cholera epidemic and the evolution of multidrug-resistant *V. cholerae* strains over the last decade, particularly in Asian countries, pose a great threat to the clinical diagnosis and treatment of this disease (Mahapatra *et al.* 2014). Kerala, a southwestern coastal state of the country, has recently experienced outbreaks of cholera. In the massive cholera outbreak reported from Malappuram and Palakkad districts in Kerala during June–July 2016, three deaths were recorded and more than a hundred people were affected (NCDC 2016). In these outbreaks, the causative organism was isolated from patients and characterized. However, isolation of pathogenic *V. cholerae* from the environment is often limited by the lack of a suitable technique to selectively enrich pathogenic strains from the vast majority of non-pathogenic strains normally found in the environment. Hence, very little information is available in the literature regarding the characterization of *V. cholerae* strains from drinking water sources implicated in the outbreak. In this context, the present study has been undertaken to isolate the toxigenic *V. cholerae* strains from well water samples from Palakkad district in Kerala (India) from where a cholera outbreak was reported and to identify their biotype and serogroup and to characterize them for virulence genes, antibiotic susceptibility profiles and genetic profiles.

MATERIALS AND METHODS

Sample collection

Pattanchery is a village in the Palakkad district of Kerala, India and it is a densely populated area with little space between houses. People live in poor quality houses. Their habitats are characterized by overcrowding, lack of basic amenities and facilities such as drinking water and sanitation. Piped municipal water supply is intermittent and several households share public water taps. People depend on well water and they share water from common wells. The main source of income is from agriculture and fish farms.

A total of 28 samples including 22 water samples (17 well water and 5 pipe water (both chlorinated)) and 6 soil samples from the surroundings of wells were collected from the households of suspected patients in Pattanchery, Palakkad district (Figure 1) from where a cholera outbreak was reported during June–July 2016. All the well water samples collected were chlorinated, given that the health authorities chlorinated all the water sources in the area of the outbreak immediately after the first report. Samples were stored in insulated thermocole boxes containing flake ice and transported aseptically to the laboratory for further analysis within 2–3 hours of collection and care was taken to avoid cross contamination.

Isolation and identification of *V. cholerae* from environmental samples

All samples collected were analysed for *V. cholerae* according to Standard Protocol (USFDA 2001). For isolation of *V. cholerae,* samples of water were directly plated (100 μL) onto thiosulfate citrate bile salts sucrose agar (Oxoid, UK), incubated at 37 °C for 18–24 h and characteristic colonies were picked, purified on tryptic soy agar (TSA) plates (Difco, USA) and identified. A part of the water (25 mL) was incubated in 225 mL alkaline peptone water (APW) at pH 7.5–8.5 (100 rpm) for 16–18 h and plated as described above. In order to prepare the soil samples for analysis, samples were added to 100 mL of distilled water and allowed to settle. An 8–10 mL amount of the slurry was centrifuged at 2,000 rpm for 8 min to remove particulate matter and 1 mL of slurry was added to 10 mL of APW (pH 8.5) for enrichment at 37 °C for 18–24 h, streaked onto TCBS agar plates and incubated overnight at 37 °C. Typical colonies were picked and purified on TSA. After purification, *V. cholerae* isolates were identified as per

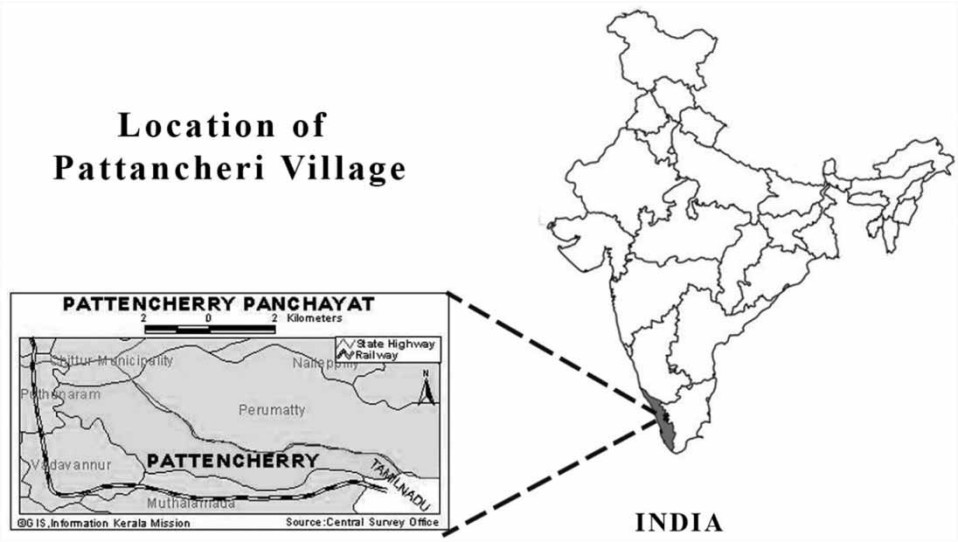

Figure 1 | Map of Kerala and location from where altered *V. cholerae* O1 Ogawa El ToR were isolated.

FDA method (Elliot *et al.* 2001) and confirmed using rapid diagnostic kit API 20E (BioMerieux SA, France).

A clinical strain, *V. cholerae* O1 El Tor Ogawa, supplied by Govt. Medical College Alappuzha, Kerala and *V. cholerae* O1 MTCC 3904 procured from Microbial Type Culture Collection (MTCC) were used as reference strains and positive controls.

Serogroup identification

All the 25 *V. cholerae* isolates were tested by slide agglutination using *V. cholerae* poly O1 antiserum and monospecific Ogawa, Inaba and Hikojima antisera (BD Difco, Maryland, USA). The *V. cholerae* isolates exhibiting a negative reaction with *V. cholerae* O1 polyvalent antiserum were typed using *V. cholerae* O139 'Bengal' antiserum (Denka Seiken, Japan). The isolates that gave negative results with both polyvalent somatic O antiserum and O139 'Bengal' antiserum were designated collectively as *V. cholerae* non-O1/non-O139.

Antimicrobial susceptibility testing

Susceptibility to various antimicrobial agents was tested by the Kirby-Bauer disc diffusion method as described by the National Committee for Clinical Laboratory Standards (CLSI 2015, 2019) using commercially available discs (Icosa GII minus, Himedia, India) with 20 antibiotics: imipenem (10 μg), tobramycin (10 μg), ofloxacin (5 μg), ciprofloxacin (5 μg), co-trimoxazole (25 μg), gentamicin (10 μg), norfloxacin (10 μg), amikacin (30 μg), levofloxacin (5 μg), augmentin (30 μg), cefoxitin (30 μg), galifluxacin (5 μg), moxifloxacin (5 μg), colistin (10 μg), ceftriazone (30 μg), nalidixic acid (30 μg), ceftazidime (30 μg), azthreonam (30 μg), nitrofurantion (300 μg) and cefpodoxime (10 μg). Sensitivity towards vibriostatic agent 2,4-diamino-6,7-diisopropylpteridine, O/129 (10 μg and 150 μg) was also tested. *Escherichia coli* American Type Culture Collection (ATCC; Manassas, VA, USA) 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 were used for the standardization of the Kirby-Bauer test for correct interpretation of the zone diameters. Isolates that are resistant to at least one agent in three or more antimicrobial categories are defined as multidrug resistant (MDR).

Molecular detection of virulence genes

All the 25 *V. cholerae* isolates were analysed for the presence of 'species specific' outer membrane protein, *ompW* gene, virulence associated genes such as *ctxA*, *ctxB*, *zot*,

ace and *VPI* genes and regulatory gene *toxR*. Serogroup-specific gene *rfb* was amplified to distinguish O1, O139, and non-O1/non-O139 *V. cholerae* strains. Genes *tcpA*, encoding the toxin coregulated pilus and *hlyA* were amplified for the discrimination of classical and El Tor biotypes. Clinical strain *V. cholerae* O1 El Tor Ogawa and *V. cholerae* O1 MTCC 3904 procured from MTCC were used as reference strains. The primers used in this study and the conditions employed are listed in Table 1.

Genomic DNA for PCR assay was extracted by the phenol–chloroform method (Ausubel *et al.* 1994) with minor modifications (Kumar *et al.* 2009). PCR assay was carried out in 25 μL of reaction buffer and was performed in AB Biosystems, Veriti 96 well thermal cycler. 50 and 100 bp molecular size markers (Gene Ruler™, Fermentas) were used for separation of the amplicons. PCR products were analysed by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, visualized under UV light and recorded with the aid of a Gel Documentation system (SYGENE, Biorad).

DNA finger printing of *V. cholerae* isolates using ERIC-PCR

Enterobacterial repetitive intergenic consensus (ERIC)-PCR was performed according to Versalovic *et al.* (1991), using ERIC primers ERIC1 R- 5′-ATGTAAG CTCCTGGGGATT-CAC-3′ and ERIC2 5′- AAGTAAGTGACTGGGGTGAGCG-3′ ERIC-PCR and amplification in Mastercycler Personal (Eppendorf, Germany). Amplification was performed in 25 μL volume containing 2.5 μL 10x reaction buffer, 1 mM each of dNTP, 5 pmol each of the forward and reverse primers, 2.5 U of Taq polymerase, 2 mM $MgCl_2$ and 10 ng of genomic DNA. PCR amplification was done as follows: denaturation at 92 °C for 45 s, annealing at 52 °C for 1 min

Table 1 | Primer sequences, annealing temperature and amplicon size used for molecular characterization by PCR assay

Gene	Primer sequence	Annealing temperature (°C)	Amplicon size (bp)	Reference
ace	GCTTATGATGGACACCCTTTA; TTTGCCCTGCGAGCGTTAAAC	55	284	Colombo *et al.* (1994)
zot	TCGCTTAACGATGGCGCGTTTT; AACCCCGTTTCACTTCTACCCA	60	947	Colombo *et al.* (1994)
hlyA	GGCAAACAGCGAAACAAATACC; CTCAGCGGGCTAATACGGTTTA	60	481	Hall *et al.* (1990)
rfb- O1	GTTTCACTGAACAGATGGG; GGTCATCTGTAAGTACAAC	55	192	Keasler *et al.* (1993)
rfb-O139	AGCCTCTTTATTACGGGTGG; GTCAAACCCGATCGTAAAGG	55	449	Keasler *et al.* (1993)
*tcp*A (El Tor)	AAGAAGTTTGTAAAAGAAGAACAC; GAAAGGACCTTCTTTCACGTTG	60	471	Keasler *et al.* (1993)
*tcp*A (Classical)	CACGATAGGAAAACCGGTCAAGAG ACCAAATGCAACGCCGAATCGAG	60	617	Keasler *et al.* (1993)
ctxA	CGGGCAGATTCTAGACCTCCTG; CGATGATCTTGGAGCATTCCCAC	60	564	Keasler *et al.* (1993)
VPI	GCAATTTAGGGGCGCGACGT CCGCTCTTTCTTGATCTGGTAG	52	680	Mukhopadhyay *et al.* (2001)
OmpW	CACCAAGAAGGTGACTTTATTGT; GAACTTATAACCACCCGCG	64	588	Nandi *et al.* (2000)
ctxB	GGTTGCTTCTCATCATCGAACCAC; GATACACATAATAGAATTAAGGAT	60	460	Olsvik *et al.* (1993)
*tox*R	TGTTCGGATTAGGACAC; TACTCACACACTTTGATGGC	60	883	Rivera *et al.* (2001)

and elongation at 70 °C for 10 min. A final elongation step at 70 °C for 20 min at the end of 35 cycles was added. The PCR amplification products were separated by electrophoresis through 1.5% agarose gel and detected by staining with ethidium bromide.

Pulsed-field gel electrophoresis (PFGE)

PFGE of *V. cholerae* isolates was performed according to PulseNet standardized protocol using restriction enzyme *Not*I (WWW.cdc.gov/pulsenet/PDF/vibrio pfge protocol-508c.pdf) on a CHEF-DR XA system (Bio-Rad Laboratories, Richmond, CA, USA). Bacteriophage lambda DNA ladder (New England Biolabs) was used as size marker. Following electrophoresis, the gel was stained with ethidium bromide and DNA bands were visualized with UV transilluminator and photographed. ERIC-PCR and PFGE were repeated twice.

DNA fingerprint analysis

DNA fingerprinting pattern of ERIC-PCR and PFGE were analysed using Gel Compar II software (Version 5.1, Applied Maths, Belgium). Cluster analysis was performed using the unweighted pair group method with arithmetic averages (UPGMA), with Dice correlation method. The position tolerance was set at 1.0% and minimum profile for each band was set at 5.0%.

RESULTS AND DISCUSSION

A total of 28 samples (17 well water, 5 pipe water, 6 soil samples) collected at the households of suspected cholera patients in Palakkad district, were analysed for the presence of *V. cholerae*. Among the 90 presumptive *V. cholerae* isolated from chlorinated water and soil and characterized biochemically as per FDA protocols (USFDA 2001), 25 isolates from three well waters were identified as *V. cholerae* and confirmed using rapid diagnostic kit API 20E. All the isolates were Voges–Proskauer (VP) test positive.

The presence of *ompW* gene confirmed the biochemical identification of all the 25 *V. cholerae* isolates. The *rfb* O1 gene (Figure 2(a)) was present in all the 25 isolates confirming that the isolates belong to O1 serogroup, and this is in agreement with the serological results. All the 25 isolates and the reference strains possessed *tcpA* El Tor (Figure 2(b)) and they were confirmed as *V. cholerae* O1 biotype El Tor and also confirmed in the phenotypic assay as VP positive, haemolysis positive and citrate positive. Serotyping of the isolates with monospecific antisera identified that the isolates belong to serotype Ogawa. *V. cholerae* O1 biotype El Tor serotype Ogawa isolates from well water samples in this study harboured *ctxA* (Figure 2(c)), *ctxB* (Figure 2(d)), *hlyA, VPI, ace, zot* and *toxR* genes. The presence of the CTX element and the Vibrio pathogenicity island in the isolates in this study indicates that they are pathogenic and epidemic strains of *V. cholerae.* The presence of *ctxB* gene of the classical biotype in the El Tor isolates indicates that they are new variants of *V. cholerae* O1 El Tor isolates that produce cholera toxin of the classical biotype. El Tor strains with classical *ctxB* gene were associated with the cholera outbreak reported from Chennai (Goel *et al.* 2010). In the earlier cholera outbreaks reported from Alappuzha and Palakkad districts in Kerala, *ctxA* and *tcpA* genes were detected in *V. cholerae* O1 EI Tor Ogawa clinical strains (Radhakutty *et al.* 1997). The detection of El Tor strains with classical *ctxB* gene in the environmental *V. cholerae* O1 in this study as well as in the outbreaks from Chennai and Odissa indicates their wide distribution in the aquatic environment of the Indian subcontinent (Goel *et al.* 2010; Pal *et al.* 2010). It has been reported that CT producing El Tor strains are now replacing the seventh pandemic El Tor strains in India and Bangladesh (Nair *et al.* 2002, 2006; Kumar *et al.* 2009). Cholera outbreaks reported in different parts of Kerala during 1996–2000 and during 2012–2016 were mainly caused by the serotype Ogawa (Radhakutty *et al.*1997; Geeta *et al.* 2005; NCDC 2016).

The phenotypic assays in this study confirmed that all the *V. cholerae* O1 isolates in this study are of the El Tor biotype background. The results of the VP test and citrate test that are used to distinguish between classical and El Tor biotypes (USFDA 2001) revealed isolates were VP and citrate positive. Genetic analysis of the *tcpA* gene that is routinely conducted to verify the biotype background of *V. cholerae* isolates (Keasler *et al.* 1993) also confirmed that the isolates belonged to El Tor biotype. The presence of *ctxB* gene of

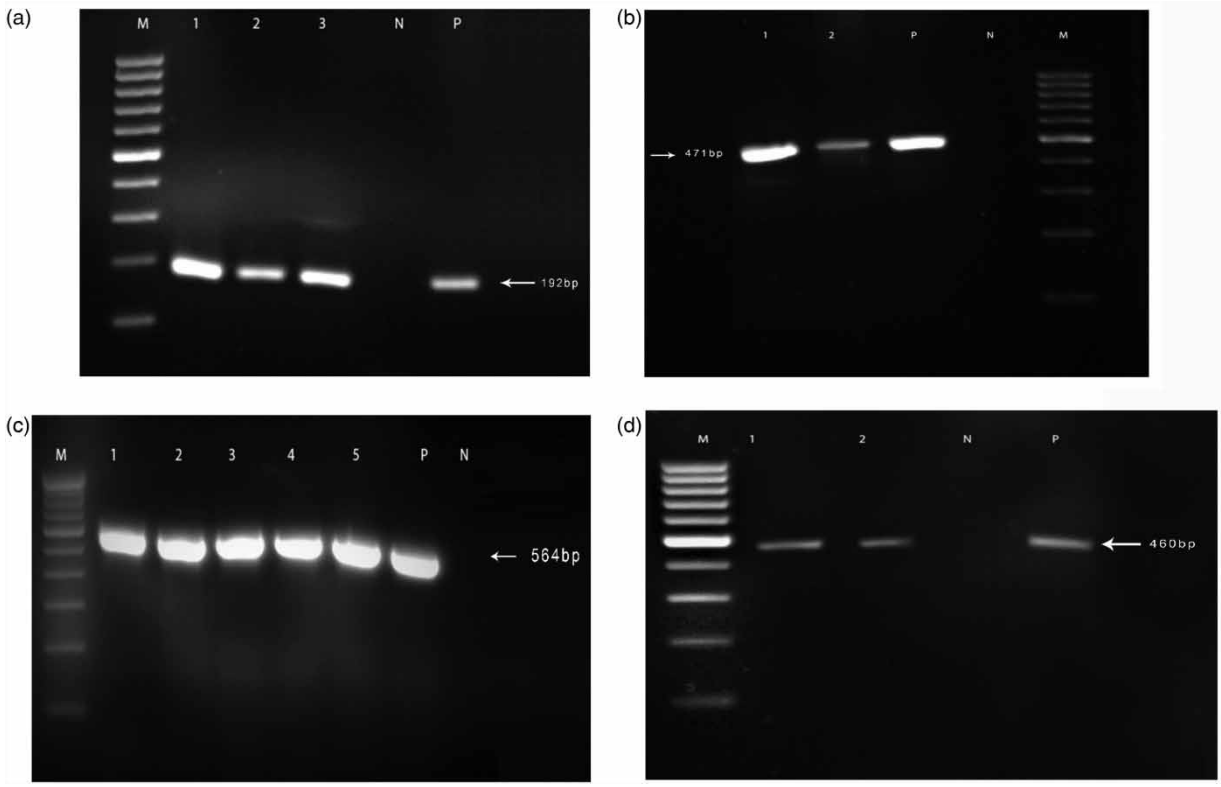

Figure 2 | (a) PCR detection of the *V. cholerae* O1 specific *rfb* O1 gene: lane M, 100 bp marker; lanes 1–3, isolated strains; lane N, negative; lane P, positive control *V. cholerae* O1 MTCC3904. (b) PCR detection of the *V. cholerae tcpA* El Tor gene: lanes 1–2, isolated strains; lane P, positive control *V. cholerae* O1 MTCC3904; lane N, negative; M, 100 bp marker. (c) PCR detection of the *V. cholerae ctxA* gene: lanes 1–5, isolated strains; lane P, positive control *V. cholerae* O1 MTCC3904; lane N, negative; M, 100 bp marker. (d) PCR detection of the *V. cholerae ctxB* gene: lanes 1–2, isolated strains; lane P, positive control *V. cholerae* O1 MTCC3904; lane N, negative; M, 100 bp marker.

the classical biotype in El Tor isolates suggests that they are variants of El Tor biotype.

In the present study, 100% resistance to augmentin, nalidixic acid, ceftriazone, co-trimoxazoe, imipenem, nitrofurantion, and ceftazidime was found in *V. cholerae* O1 altered El Tor Ogawa strains and they were multidrug resistant, suggesting these drugs could not be used for clinical purposes. Similarly, 25 isolates were found to be resistant towards vibriostatic agent 2,4-diamino-6,7-diisopropylpteridine (O/129). Das *et al.* (2011) and Shrestha *et al.* (2015) also reported the resistance of *V. cholerae* O1 El Tor against nalidixic acid and cotrimoxazole.

However, all the isolates were sensitive to colistin, ciprofloxacin, moxifloxacin, cefoxitin, azthreonam, gentamicin, gallifluxacin, ofloxacin, tobramycin, norfloxacin, amikacin and levofloxacin. *V. cholerae* O1 El Tor Ogawa isolates possessing the *ctxB* gene of the classical biotype obtained in the present study were sensitive to ciprofloxacin and norfloxacin, as reported by Pal *et al.* (2010), for environmental strains of the El Tor variant with the *ctxB* gene of the classical biotype. However, a progressive increasing trend of antibiotic resistance in clinical strains of *V. cholerae* O1 El Tor towards common fluoroquinolone, i.e., ciprofloxacin and norfloxacin, has been reported since 1996 (Garg *et al.* 2001; Krishna *et al.* 2002; Goel *et al.* 2010; Pal *et al.* 2010). Similar to our observation, resistance to nalidixic acid, co-trimoxazole and furazolidone were also reported in *V. cholerae* O1 El Tor Ogawa isolates carrying the *ctxB* gene of classical biotype implicated in the cholera outbreak from Odissa and Solapur (Pal *et al.* 2010; Goel *et al.* 2011).

All the 25 *V. cholerae* O1 isolates were characterized by ERIC-PCR and PFGE to study their genetic relatedness. ERIC-PCR with genomic DNA of *V. cholerae* isolates resulted in amplification of multiple fragments of DNA ranging between 100 bp and 2,000 bp. ERIC-PCR finger prints grouped the isolates into two clusters (C1 and C2) (Figure 3).

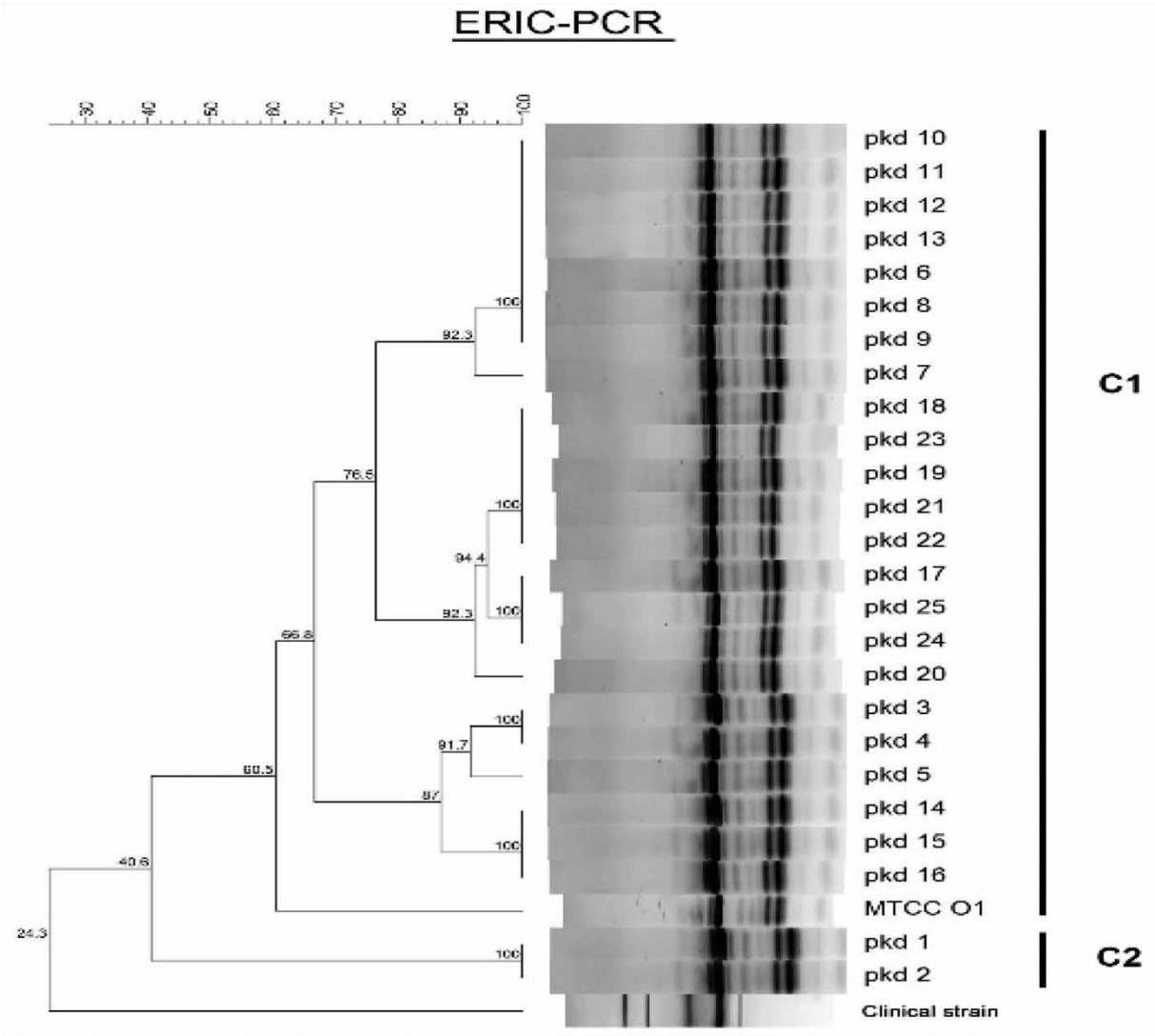

Figure 3 | Dendrograms showing genomic fingerprints of altered *V. cholerae* O1 El Tor Ogawa isolated from well water, Kerala, India generated by ERIC-PCR based on unweighted pair-group method with arithmetic means (UPGMA) using Gel Compar II software, version 5.1 (Applied-Maths, St-Martens-Latem, Belgium).

C1 formed the largest cluster with 23 isolates and the other 2 isolates belonged to C2 cluster. The clinical strains formed separate clusters. ERIC sequences are 126 bp imperfect palindromes that occur in multiple copies in the genomes of Vibrio, located near the haemolysin gene, and are less complex but more discriminative (Waturangi *et al.* 2012). In ERIC-PCR, toxigenic *V. cholerae* have shown multiple fragments of DNA ranging between 0.25 and 1.8 kb (Goel *et al.* 2010).

The analysis of PFGE fingerprints revealed that the isolates were grouped into two clusters (P1 and P2) (Figure 4). Seventeen isolates formed one cluster (P1) and eight isolates formed another cluster (P2). The clinical reference strains of O1 El Tor and O1 MTCC strain formed separate clusters different from P1 and P2. The results indicate that El Tor biotype isolates do not share similarities in their genetic traits with the clinical El Tor type strain used in the study. The distribution of isolates into two clusters suggest that two clones of *V. cholerae* might have been implicated in the Palakkad outbreak. This study indicates that continuous monitoring of *V. cholerae* strains associated with outbreaks is needed to understand the evolution of pathogen and disease patterns.

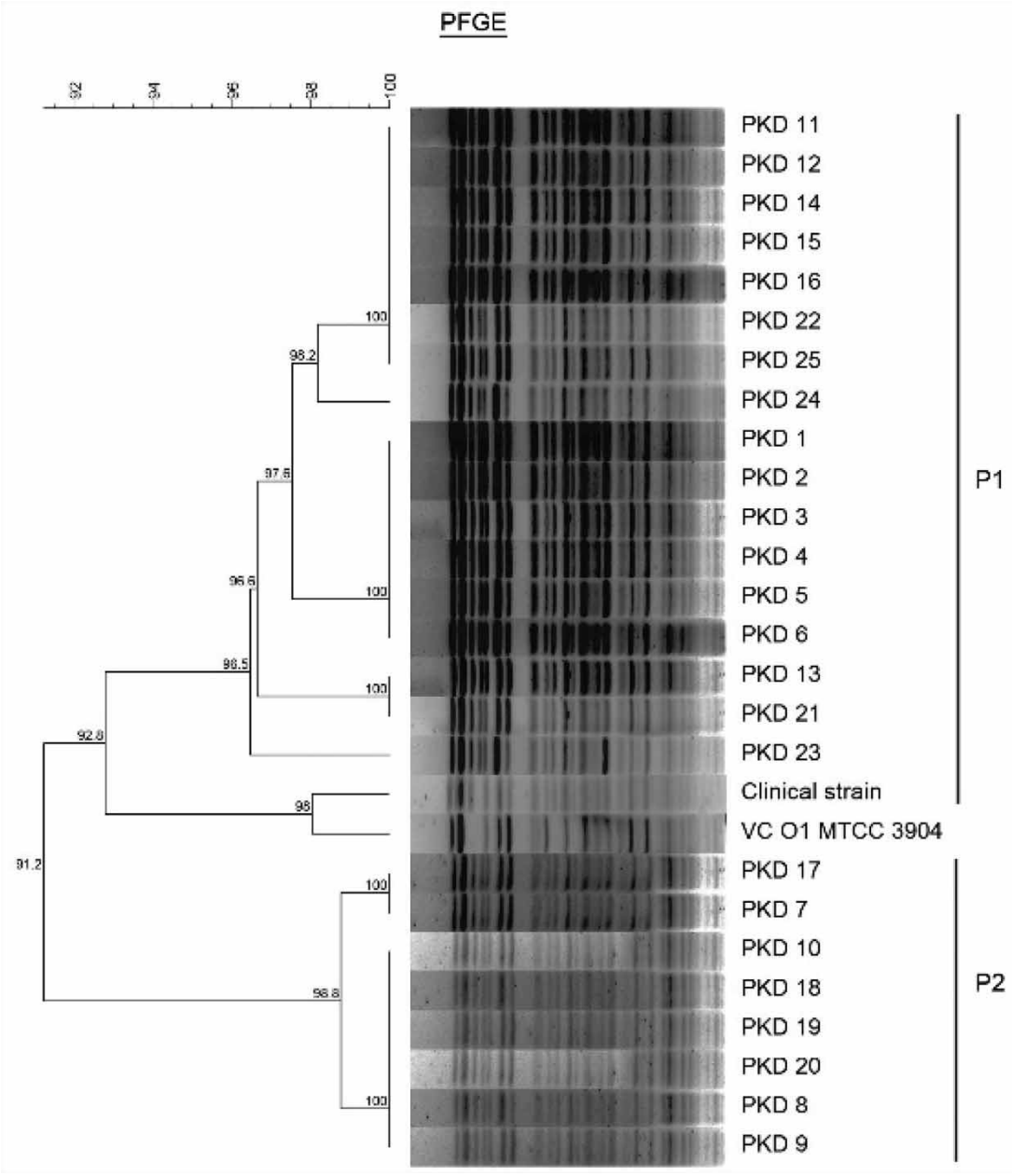

Figure 4 | Dendrogram showing genomic fingerprint pattern of a representative altered *V. cholerae* O1 El Tor Ogawa, isolated from well water in Kerala, India. The dendrogram was generated by Dice similarity coefficient and UPGMA clustering methods by using PFGE images of *NotI* digested genomic DNA.

CONCLUSION AND RECOMMENDATION

This study revealed the presence of a multidrug-resistant new variant of *V. cholerae* El Tor Ogawa in well water with CTX genes. To our knowledge, this is the first report regarding the isolation of a multidrug-resistant toxigenic new variant of *V. cholerae* O1 El Tor Ogawa from well water in Kerala. The study suggests that contaminated well water may be incriminated in the outbreak from Palakkad district in Kerala. The pathogens released from point and diffuse sources from the outbreak area get transported from upstream sources to coastal waters, thereby increasing

the chances of spread of the disease to other areas. For enhanced control and to reduce the potential risks of the dreadful disease cholera, the sanitation process has to be improved in the outbreak area and, at the same time, the quality of drinking water need to be monitored frequently. The use of multiple typing methods would permit a more precise characterization of the genetic diversity to better understand the evolution of pathogen and disease patterns.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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