Phenotypic and genetic characteristics of *Vibrio cholerae* O1 carrying Haitian *ctxB* and attributes of classical and El Tor biotypes isolated from Silvassa, India

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*Vibrio cholerae* O1 biotype El Tor, the causative agent of the seventh pandemic, has recently been replaced by strains carrying classical and Haitian *ctxB* in India, Haiti and other parts of the world. We conducted phenotypic and genetic tests to characterize *V. cholerae* O1 isolated between 2012 and 2014 from Silvassa, India, to examine the presence of virulence and regulatory genes, seventh pandemic marker, *ctxB* type and biofilm formation and to study genomic diversity. Of the 59 *V. cholerae* O1, eight isolates belong to El Tor prototype, one to classical prototype and the remaining isolates have attributes of both classical and El Tor biotypes. PCR and *ctxB* gene sequencing revealed the presence of classical *ctxB* in four strains and Haitian *ctxB* in 55 isolates; indicating that isolates were either an El Tor or hybrid variant. All isolates carried virulence, regulatory, adherence, *Vibrio* seventh pandemic pathogenicity island I and seventh pandemic group-specific marker VC2346, in addition to tcpA<sup>ET</sup> and rstR<sup>ET</sup>, the features of seventh pandemic strains, and produced cholera toxin and biofilm. PFGE analysis showed that the majority of isolates are clonal and belong to fingerprint pattern A; however, pattern B is unrelated and patterns C and D are distinct, suggesting considerable diversity in the genomic content among them. These data thus show that isolates from Silvassa are genetically diverse and that Haitian *ctxB* and hybrid phenotypes are undergoing global dissemination.

INTRODUCTION

*Vibrio cholerae*, an autochthonous inhabitant of water and the estuarine system, is the causative agent of the disease cholera (Colwell et al., 1977). *V. cholerae* is classified into more than 200 serogroups from variations in O-antigen structure, of which only the O1 and O139 serogroups are reported to have caused epidemics. *V. cholerae*, classified into two biotypes (the classical and El Tor biotypes) (Kay et al., 1994; WHO, 1987), differs in two major genomic regions, namely, *Vibrio* seventh pandemic pathogenicity island (VSP-I) and VSP-II that are unique to the seventh pandemic El Tor biotype (Dziejman et al., 2002). Additionally, variations in genotype-specific genes tcpA (toxin co-regulated pilus), *ctxB* (cholera toxin B) and rstR (repeat sequence transcriptional regulator), along with the epitope of one of the two subunits of cholera toxin (CT) (CTB typing), are used as a marker to differentiate biotypes and to track the origin of strains (Nair et al., 2006; Safa et al., 2010).

The severity of cholera is associated with production of CT which is encoded by a gene present on the 6.9 kb CTXΦ prophage, mostly located on the large chromosome (Waldor & Mekalanos, 1996). Although classical and El Tor strains have identical sequences of the *ctxA* gene, substitution of two nucleotides at positions 113 and 203 in the *ctxB* gene results in the substitution of two amino acids that differentiate classical and El Tor strains (Olsvik et al., 1993; Popovic et al., 1994). Earlier studies have shown remarkable changes in phenotypic and genetic characteristics of El Tor biotype...
causing cholera in Asian countries (Nair et al., 2002). These authors further showed that El Tor strains causing acute diarrhoea in Bangladesh were hybrid because they possessed phenotypic and genetic traits of classical biotype against an El Tor background (Nair et al., 2002). Based on phenotypic and genetic characteristics, the El Tor variants are classified into four groups: Matlab variants that are categorized into three subtypes, altered El Tor variant, Mozambique and Hybrid El Tor variant (Nair et al., 2002, 2006; Raychoudhuri et al., 2008). The new variant of El Tor possessing the ctxB allele, which had an additional mutation in classical ctxB at 20 positions designated as Haitian ctxB, genotype 7, was reported from the recent epidemic in Haiti (Chin et al., 2011; Hasan et al., 2012; Talkington et al., 2011). This new type of ctxB was first reported by Goel et al. (2008) in V. cholerae strains isolated from a cholera outbreak in Kalahandi, Odisha, India. In a retrospective study, it was shown that Haitian ctxB first appeared in Kolkata, India, in April 2006 (Naha et al. 2012). It was speculated that Haitian ctxB may have originated from Kolkata and then disseminated to the neighbouring regions like Odisha, and other places, and then via Nepal to Haiti (Hendriksen et al., 2011; Naha et al., 2012; Harroux et al., 2011).

In this study, we conducted phenotype and genetic tests to characterize V. cholerae O1 isolates from cholera outbreaks between 2012 and 2014 in Silvassa, western India, to examine the presence of virulence and regulatory genes, seventh pandemic markers, type of ctxB, production of CT and capability of biofilm formation. PFGE was used to study genomic diversity among V. cholerae O1 strains.

METHODS

Bacterial strains. A total of 59 V. cholerae O1 isolates from diarrhoeal patients admitted to Shri Vinoba Bhave Civil Hospital, Silvassa, for which stool samples were processed and identified in the Department of Microbiology, Shri Vinoba Bhave Civil Hospital, Silvassa, Union Territory of Dadra and Nagar Haveli, India, between July 2012 and December 2014 (21 strains isolated in 2012, 18 strains isolated in 2013 and 20 strains isolated in 2014), were included in the study. All isolates were examined for the oxidase reaction, and identities of isolates were confirmed by serogrouping using growth from triple sugar iron agar slant with polyvalent O1 and monospecific Inaba and Ogawa anti-sera (Becton Dickinson). Biotypes were determined as described previously (Kay et al., 1994; WHO, 1987). V. cholerae O1 classical strain 569B and El Tor strain N16961 were used as controls. All isolates were maintained in tryptic soy broth (Difco) supplemented with 30% glycerol at −80°C or in Luria–Bertani agar stab culture at room temperature. The details of the isolates and place and year of isolation are shown in Table S1 (available in the online Supplementary Material).

Antibiotic susceptibility testing. Isolates were tested for antimicrobial susceptibility by the method of Bauer et al. (1966) on Mueller–Hinton agar using antibiotic discs (Hi-Media Laboratories) with ampicillin (10 μg), chloramphenicol (30 μg), cefotaxime (30 μg), ciprofloxacin (5 μg), cephalin (30 μg), co-trimoxazole (25 μg), furazolidone (100 μg), gentamycin (10 μg), neomycin (30 μg), norfloxacin (10 μg), polymyxin B (50 U), streptomycin (30 μg), sulfamethoxazole (300 μg), nalidixic acid (10 μg), trimethoprim (5 μg) and tetracycline (30 μg). Escherichia coli strain ATCC 25922 and Staphylococcus aureus strain ATCC 29213 were used as sensitive controls, and results were interpreted according to CLSI guidelines, except for resistance to furazolidone (CLSI, 2010).

PCR assays. V. cholerae strains grown overnight at 37°C in Luria–Bertan broth (Difco, Becton-Dickinson) were boiled for 10 min and stored at −20°C until use. The bacterial cell lysates were used as template DNA in all PCR assays. The presence of antibiotic resistance genes and SXT genetic element was determined by multiplex PCR (Rama-chandran et al., 2007). The presence of virulence and regulatory genes ctxA, zot, ace, tcpA, ompU and toxR was determined by a hexaplex PCR assay using primers as previously published (Singh et al., 2002). Single PCR assays were used to detect the genes tcpI, aeb, ctxA, rstC and hlyA (Singh et al., 2001). Other PCR assays were also used to examine the presence of rstR, VSP-1 and seventh pandemic group-specific marker V2346 and to examine for insertion of CTXφ and VSP-1 in chromosomes (Grim et al., 2010; Maiti et al., 2006; Mantri et al., 2010). Amplification was performed using oligonucleotides (GCC Biotech) as shown in Table S2. Amplified products were separated on agarose gel, stained with ethidium bromide and visualized in Fluoro-S-Multilmage (Bio-Rad).

Double-mismatch amplification mutation assay-PCR. We performed double-mismatch amplification mutation assay (DMAMA)-PCR using biotype-specific primers Rv-cla (5′-CCGTGTACCTCTACCTCGAAACG-3′), ctxB-3 (5′-GGTTTTATCTCTCAGCAATTGG-3′) and ctxB-4 (5′-GGTTTTATCTCTCAGCAATTGG C-3′) for detection of classical and Haitian ctxB (Naha et al., 2012). DMAMA-PCR was also performed using primers Fw-com (5′-ACTATCTTCTTAGATTGACACATGGG-3′) and Re-elt (5′-CTCCGTGATCTTACTCTTAGAAAAC-3′) to determine the presence of El Tor ctxB in these isolates (Morita et al., 2008).

Sequencing of the ctxB gene. The DNA sequence of a rstR gene and ctxB was amplified, using the forward primer of rstR-2w and reverse primer of ctxB that yielded an amplicon of ~6.9 kb. This PCR product was then used as a template for nested PCR (Mantri et al., 2010). PCR products were purified using EXPO (USB), and both strands were sequenced using Life Technology ABI sequencer model 3537 with a BigDye terminator kit at the sequencing facility of the Institute of Life Sciences, Bhubaneswar. The sequences of ctxB gene for classical and El Tor reference strains were retrieved from GenBank (NCBI). The deduced amino acid sequences of ctxB from all isolates were aligned using ClustalW.

Assay for CT production. GM1-ganglioside-dependent enzyme determined the ability of ctx-gene-positive V. cholerae O1 strain to produce CT in vitro by ELISA (GM1-ELISA) (Swennerholm & Holmgren, 1978).

Assay for biofilm formation. Biofilm in V. cholerae O1 isolates was quantified by the method of O’Toole et al. (1999). Formation of biofilm was measured at OD570 nm using an ELISA Reader (BioTek Synergy HT) using V. cholerae O1 strain N16961 and E. coli strain DH5α as positive and negative controls, respectively. Biofilm formation experiments were done in triplicate, and data showing values P>0.05 were omitted.

PFGE. Isolates were analysed using a PulseNet standardized PFGE protocol for V. cholerae (Cooper et al., 2006) with NotI restriction enzymes (NEB). PFGE was performed using a Clamped Homogeneous Electric Fields (CHEF) Mapper (Bio-Rad). Digested fragments ranging in size from 20 to 300 kb were separated in 1% Pulse Field Certified Agarose (Bio-Rad) prepared in 0.5x Tris Borate EDTA (TBE) buffer. The running conditions used for separation were as described in the PulseNet protocol (http://www.cdc.gov/pulsenet/PDF/vibrio_pfge_protocol-508c.pdf). The gel was stained with ethidium bromide and photographed using a gel documentation system (Bio-Rad).
Image analysis. After visualization, the fingerprint profile in the PFGE gel was analysed using the computer software package Bionumerics (7.1 versions) (Applied Maths). The fingerprinting pattern, after background subtraction and gel normalization, was subjected to typing by band-based similarity Dice coefficient, which provides a quantitative assessment of strain similarity. Using 1.5% optimization, 1.5% tolerance and threshold linkage value of >95% similarity matrix, strains were clustered together. Clustering was done based on unweighted pair group methods using average linkages as recommended by the software manufacturer, and results are graphically represented as a dendrogram.

RESULTS

Biochemical characteristics

All isolates were identified as V. cholerae belonging to serogroup O1, serotype Ogawa, which was also confirmed by O1/O139, ctxA PCR (Hoshino et al., 1998). Eight isolates comprising four strains isolated in 2012 and two each isolated in 2013 and 2014 were prototype El Tor that showed positive results for chick cell agglutination (CCA‘), Voges–Praskauer (VP) test (VP‘) and resistance to polymyxin B (PB‘), identical to reference strain El Tor N16961. One strain isolated in 2014 showed phenotypic traits [negative results for chick cell agglutination (CCA‘/C0), VP test (VP‘/C0)] of the classical prototype, identical to the classical reference strain 569B. However, the majority of isolates showed a variable reaction to sheep erythrocyte haemolysis. The remaining isolates showed mixed phenotype(s) referred hereto as hybrid prototype(s), irrespective of year of isolation. The results of the combination of phenotypic tests are shown in Table 1.

Antibiotic resistance pattern

All of the V. cholerae O1 strains were resistant to furazolidone and nalidixic acid, irrespective of year of isolation. However, 57 strains, comprising 19 strains isolated in 2012, 18 strains isolated in 2013 and 20 strains isolated in 2014, were resistant to co-trimoxazole, streptomycin (except for eight strains isolated in 2014), sulfafurazole and trimethoprim, of which two strains isolated in 2012 showed additional resistance to trimethoprim (Fig. 1). Two strains isolated in 2014 were resistant to rifampicin. All strains were susceptible to the remaining antibiotics tested. We observed increased sensitivity towards ampicillin among O1 strains isolated in 2012, 2013 and 2014. It was found that although 8 of the 21 strains isolated in 2012 showed resistance to ampicillin, only one of the 18 strains isolated in 2013 showed resistance to ampicillin. All strains isolated in 2014 were susceptible to ampicillin (Fig. 1). All strains, barring two, showing negative results by PCR for sulII and strB genes, amplified a portion of 626 bp of sulII, 278 bp of dfrA1, 515 bp of strB genes and 1035 bp of intSXT (data not shown).

Table 1. Phenotypic and genetic characteristics and proposed biotype(s) of V. cholerae O1 isolates from 2012 to 2014 in Silvassa, India, included in this study

<table>
<thead>
<tr>
<th>Biotype</th>
<th>Isolate designation (no. of isolates)</th>
<th>Year of isolation</th>
<th>Phenotype</th>
<th>Genotype</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>CCA</td>
<td>Hly</td>
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<tr>
<td>El Tor variant I</td>
<td>DN3, DN15, DN16 (3)</td>
<td>2012</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Hybrid variant I</td>
<td>DN1 (1)</td>
<td>2012</td>
<td>+</td>
<td>+</td>
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<td></td>
<td>DN37, DN38 (2)</td>
<td>2013</td>
<td></td>
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<tr>
<td></td>
<td>DN47, DN60 (2)</td>
<td>2014</td>
<td></td>
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<tr>
<td>El Tor variant II</td>
<td>DN56 (1)</td>
<td>2014</td>
<td></td>
<td></td>
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<tr>
<td>Hybrid variant II</td>
<td>DN2, DN12 (2)</td>
<td>2012</td>
<td></td>
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<td></td>
<td>DN25 (1)</td>
<td>2013</td>
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<td></td>
<td>DN64 (1)</td>
<td>2014</td>
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<td>Hybrid variant III</td>
<td>DN4, DN14 (2)</td>
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<td>Hybrid variant IV</td>
<td>DN5, DN6, DN9, DN10, DN18, DN21 (6)</td>
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<td>+</td>
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<tr>
<td></td>
<td>DN26, DN28, DN32, DN33, DN39, DN42, DN44 (7)</td>
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<td>DN65, DN72 (2)</td>
<td>2014</td>
<td></td>
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<td>Hybrid variant V</td>
<td>DN7, DN19 (2)</td>
<td>2012</td>
<td></td>
<td>+</td>
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<tr>
<td>Hybrid variant VI</td>
<td>DN13 (1)</td>
<td>2012</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>DN52, DN63, DN73 (3)</td>
<td>2014</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hybrid variant VII</td>
<td>DN17 (1)</td>
<td>2012</td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

CCA, chick cell erythrocyte agglutination; +, agglutination; −, no agglutination; Hly, sheep blood haemolysis; +, haemolysis; −, no haemolysis; PB, polymyxin B; S, sensitive; R, resistant; VP, Voges–Praskauer; +, positive test; −, negative test; ctxB, cholera toxin subunit-B; rstR, repeat sequence transcriptional regulator; H, Haitian ctxB; E, El Tor ctxB; C, classical ctxB.
Presence of virulence and regulatory genes

Analysis of virulence and regulatory genes showed that all the 59 isolates of *V. cholerae*, barring two, showing a negative result for toxR harboured ctxA, zot, ace, tcpA (El Tor), ompU and toxR genes. All these strains also produced cholera toxin in the range 0.02–20.731 pg ml⁻¹ (data not shown). All isolates amplified tcpI, adfB, hlyA<sub>ET</sub>, hlyA<sub>Clas</sub> and rstC genes in PCR assays. Determination of the presence of rstR and location of CTX<sub>F</sub> showed that all isolates carried the rst<sub>R</sub><sub>ET</sub> and CTX<sub>F</sub> located on the large chromosome, except one strain isolated in 2012 that contained a second copy of CTX<sub>F</sub> on the small chromosome (data not shown).

DMAMA-PCR and nucleotide sequencing

DMAMA-PCR assay yielded classical ctxB in four isolates; however, amplification of Haitian ctxB was shown by 55 strains of *V. cholerae* O1 (data not shown). This finding was confirmed by DNA sequencing of the ctxB amplicon obtained with primers ctxB-F and ctxB-R by the PCR method described earlier (Mantri et al., 2010). The deduced amino acid sequences obtained with Silvassa isolates were aligned with the ctxB sequences of reference strains of El Tor N16961 and classical 569B. Four isolates showed amino acid sequences of CT-B identical to classical reference strains of DMAMA-PCR and nucleotide sequencing. Determination of the presence of ctxB obtained in this study were deposited in GenBank under accession numbers KJ730027 to KJ730047, KP037226 to KP037243 and KT734638 to KT734657.

VSP-I pathogenicity island and chromosomal insertion sites

Seventh pandemic strains including *V. cholerae* O1 strains N16961 and MJ-1236 were reported to carry VSP-I inserted between VC0174 and VC0186 of the large chromosome. However, *V. cholerae* O1 MJ-1236 also harboured a second copy of VSP-I inserted between genes VCA0095 and VCA0096 on the small chromosome, indicating that, in strain MJ-1236, VSP-I was integrated into both chromosomes (Grim et al., 2010). However, in this study, all isolates amplified a portion of 331 bp targeted to VC0180 and VC0181 and 584 bp targeted to VC0175 (data not shown), indicating the presence of VSP-I. All isolates also gave a positive result by PCR for seventh pandemic group-specific marker VC2346 amplifying a portion of the 405-bp amplicon, indicating that all isolates belonged to seventh pandemic strains (data not shown).

Strains failed to yield any amplicon when the VSP was inserted between VC0175 and VC0186 on chromosome-I and between VCA0695 and VCA0696 on chromosome-II. In this study, all isolates, except *V. cholerae* O1 classical strain 569B, failed to produce any amplicon using target VC0175 and VC0186 but produced an amplicon of 957 bp using target VCA0695 and VCA0696, indicating that VSP-I was integrated on the large chromosome (data not shown).

Estimation of biofilm formation

Twenty-three strains produced weak biofilm and 22 isolates produced good biofilm. The remaining isolates produced excellent biofilm (data not shown). These data show that the majority of O1 strains have the ability to form biofilm, irrespective of year of isolation.

![Fig. 1. *V. cholerae* O1 strains showing resistance to antibiotics and isolated during 2012–2014 from Silvassa, India. A, ampicillin; C, ciprofloxacin; Co, co-trimoxazole; F, furazolidone; G, gentamycin; Na, nalidixic acid; N, neomycin; Nx, norfloxacin; Pb, polymyxin B; R, rifampicin; S, streptomycin; Sf, sulfafurazole; T, tetracycline; Tr, trimethoprim. Black bars indicate resistance, white bars indicate sensitive.](http://jmm.microbiologyresearch.org)
carried classical which eight had phenotypic traits of El Tor; three isolates of these strains. Serological tests confirmed that all isolates showed mixed phenotype using fingerprint pattern A (cluster B). Also, three other strains along with classical strain 569B showed a closely related pattern grouped with cluster B. Moreover, two isolates that showed entirely distinct fingerprint patterns C and D were not reported earlier among V. cholerae O1 carrying the Haitian ctxB. The reference El Tor strain N16961 with El Tor phenotype, therefore, will play an important role in understanding the epidemiology and severity of infection of emerging V. cholerae strains and will also help in the proper designation of V. cholerae O1 strains. Furthermore, the existence of such strains will also have implications for the development of a cholera vaccine.

Haemolysis was used as a virulence factor in Vibrio spp. and also to distinguish biotypes (Richardson et al., 1986). Genetically classical biotype possesses a truncated hlyA locus, and many El Tor strains do not produce haemolysis on blood agar plates. These strains possess the hlyA locus of classical and El Tor; many isolates do not cause haemolysis on blood agar plates, thus showing variation in haemolysis. This observation corroborates the findings of other workers who also reported that haemolysis cannot be used as a reliable marker for either strain characterization or biotyping (Raychoudhuri et al., 2008; Singh et al., 2001).

V. cholerae O1 strains isolated from Haiti and other regions around the world showed consistent resistance to sulfamethoxazole and streptomycin, but were susceptible to tetracycline (Talkington et al., 2011). Similarly, all isolates used in this study showed phenotypic resistance to trimethoprim but showed variable resistance to co-trimoxazole, sulfamethoxazole and streptomycin. However, one isolate showing resistance to tetracycline and two strains to rifampicin are a matter of concern. The presence of a 62 kb self-transmissible transposon-like element (SXT element) encoding resistance to sulfamethoxazole, trimethoprim and streptomycin in V. cholerae O1 strains isolated after an O139 epidemic has been reported (Waldor et al., 1996). When strains were tested for the presence of intSXT, sulII, strB and dfrA1/dfr18 genes encoding for SXT, sulfamethoxazole, streptomycin and trimethoprim, all showed positive

**DISCUSSION**

The emergence of an altered El Tor carrying genes encoding for ctx and possessing classical biotype has been reported (Evins et al., 1995; Nair et al., 2002). This observation is interesting because the classical biotype caused the sixth pandemic before being replaced by the El Tor biotype, which is considered extinct. It was noted that altered El Tor strains replaced the prototype of the seventh pandemic El Tor in Asia (Evins et al., 1995; Nair et al., 2002) and Africa (Lizarra-Partida et al., 2009; Morita et al., 2008). However, the Haitian ctxB variant of V. cholerae was reported to cause epidemics in Asia and Africa (Bhattacharya et al., 2016; Goel et al., 2008; Naha et al., 2012; Rashed et al., 2012; Talkington et al., 2011). Allelic variations in ctxB, tcpA and rstR are used as a marker to differentiate and characterize CTXΦ types and track the origin of strains. Serological tests confirmed that all isolates of V. cholerae belonged to serogroup O1 Ogawa, of which eight had phenotypic traits of El Tor; three isolates carried classical ctxB and five Haitian ctxB, and one the classical biotype, in addition to ctxB. However, the majority of isolates showing mixed phenotype traits had Haitian ctxB. Several workers also reported that strains showing mixed phenotype but genetically containing tcpA<sup>ET</sup> and rstR<sup>ET</sup> were designated hybrid El Tor variant, and those strains that showed typical features of El Tor biotype but carried the classical ctxB or classical biotype but carried the El Tor ctxB were designated as El Tor variant (Na-Ubol et al., 2011; Raychoudhuri et al., 2009; Safa et al., 2010). To the best of our knowledge, we are the first to report the presence of V. cholerae O1 with mixed phenotypes possessing the Haitian ctxB in Silvassa, western India, herewith referred to as a hybrid variant. The biotyping scheme, therefore, will play an important role in understanding the epidemiology and severity of infection of emerging V. cholerae strains and will also help in the proper designation of V. cholerae O1 strains. Furthermore, the existence of such strains will also have implications for the development of a cholera vaccine.

Fig. 2. Amino acid sequence alignment of ctxB gene fragment obtained with two strains each of V. cholerae O1 isolated in 2012, 2013 and 2014 from Silvassa, western India. Other isolates not shown here revealed amino acid sequences identical to that of Haitian ctxB. Dots represent identical amino acids.

**PFGE**

NotI-digested genomic DNA was performed to determine the genetic fingerprint of V. cholerae O1 isolated between 2012 and 2014 in Silvassa, western India. The number of fragments ranged from 22 to 24, and the molecular size of the PFGE restriction fragment ranged from 25 to 700 kb (Fig. 3). The dendrogram generated from the PFGE fingerprint patterns obtained with NotI-digested genomic DNA separated 48 isolates with Haitian ctxB (genotype 7) showing fingerprint pattern A (cluster A). Four isolates with classical ctxB (genotype 1), along with one isolate carrying Haitian ctxB showing an identical pattern, were clustered together (cluster B). Also, three other strains along with classical strain 569B showed a closely related pattern grouped with cluster B. Moreover, two isolates that showed entirely distinct fingerprint patterns C and D were not reported earlier among V. cholerae O1 carrying the Haitian ctxB. The reference El Tor strain N16961 with El Tor phenotype showed an entirely distinct and unrelated pattern E.
The dendrogram was prepared using 1.5% optimization, 1.5% tolerance and >95% similarity matrix of the Dice similarity coefficient; 1.5% optimization and tolerance are shown in Fig. 3. The dendrogram showing the genomic fingerprint pattern of *V. cholerae* O1 strains isolated from Silvassa, western India. The dendrogram was prepared using 1.5% optimization, 1.5% tolerance and >95% similarity matrix of the Dice similarity coefficient.
results for intSXT and dfrA1, whereas 57 strains were positive for sulII and 49 for strB genes (data not shown). We found good correlation between phenotypic resistance and the presence of respective genes among the strains.

The presence of VSP-I, VSP-I variant and VC2346 in O1 identified the seventh pandemic V. cholerae O1 strains in circulation (Grim et al., 2010). Variation in VSP-I and the presence or absence of VC2346 and its integration into the chromosome determine the evolution and emergence of new strains. The presence of toxin co-regulated pilus, colonization factor, CTX<sup>ET</sup>Φ, VSP-I, VSP-I variant and VC2346 marker is the feature of an epidemic strain. Like seventh pandemic strains, the present isolates contained VSP-I, and seventh pandemic group-specific marker VC2346 and VSP-I and CTX<sup>ET</sup>Φ inserted on the large chromosome, indicating the migration and circulation of O1 isolates in Silvassa, western India. These isolates also possess other characteristics of seventh pandemic strains, like tcp<sup>ET</sup>A and rst<sup>ET</sup>A, but differ in their phenotype and ctxB type. These data thus indicate that seventh pandemic strains may still be undergoing gradual and constant change to replace El Tor with a hybrid variant in that part of the world.

The majority of V. cholerae O1 strains presented an overall banding pattern characteristic of the Haitian ctxB variant reported from Kolkata and Haiti (Naha et al., 2012; Talkington et al., 2011). We did not find any correlation between fingerprinting patterns and phenotypes of V. cholerae. Overall, PFGE analysis revealed that El Tor and/or hybrid variant have four fingerprinting patterns, patterns A–D, with major pattern A having nine different fingerprint patterns, A1–A9. These findings are similar to those reporting different PFGE patterns among strains from Kolkata and Haiti carrying Haitian ctxB (Naha et al., 2012; Talkington et al., 2011). Like Kolkata isolates, Silvassa isolates with classical ctxB also showed a different pattern designated as pattern B, and a close relationship with classical strain S69B. The presence of two new pulsotypes indicates the existence of continued evolution and emergence of strains. It is clear from this study that there is consistency in the dominance of a particular PFGE pattern in V. cholerae that has all the characteristics of a pathogenic strain. However, the distinct PFGE patterns also indicate considerable diversity in the genomic content between them. These observations are similar to those reporting diversity among V. cholerae O1 strains carrying Haitian ctxB, but with the dominance of a particular PFGE pattern in Kolkata (Naha et al., 2012) and Haiti (Talkington et al., 2011). Therefore, it is speculated that Haitian ctxB variant may have originated from Kolkata, then disseminated to Haiti via Nepal and to Silvassa, although certain changes in phenotypes occurred during this process. These phenotypic and genetic characteristics led us to hypothesize that hybrid variant increases their relative fitness, perhaps as a consequence of increased pathogenicity, and caused cholera outbreaks.

Although strains from Haiti genetically belong to El Tor biotype but carried the ctxB7 allele, this allele was first seen in 2007 from an outbreak in Odisha, India (Goel et al., 2008). El Tor strains carrying ctxB<sup>ET</sup>7 have the potential to cause severe diarrhoea have been reported (Na-Ubol et al., 2011; Safa et al., 2010). These data also show that excepting a few isolates that had the typical characteristic of El Tor or classical, mixed phenotypes were shown by the majority of isolates, indicating the evolution and appearance of a hybrid variant. This observation thus indicates that genetic exchange between divergent bacterial lineages may have contributed to the emergence of hybrid variant carrying Haitian ctxB. This phenomenon would also have impact on treatment, public health measures as well as vaccine development against cholera; therefore, rapid diagnostic methods are needed for continuous monitoring of future outbreaks.

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