Molecular characterization of *Vibrio cholerae* O1 strains circulating in Assam: a north eastern state of India

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ABSTRACT

**Background and Objectives:** Information on the genetic epidemiology of cholera in Assam, a northeastern state of India is lacking despite cholera being a major public health problem. The study aimed to determine the virulence genes and genes encoding antibiotic resistance in *Vibrio cholerae* isolates and to determine the prevalent genotypes based on the presence or absence of the virulence genes and ctxB genotype.

**Materials and Methods:** Twenty-five *V. cholerae* strains were subjected to conventional biotyping and serotyping followed by multiplex PCR to detect ctxA, ctxB, zot, ace, O1rfb, tcpA, ompU, ompW, rtxC, hly and toxR and antibiotic resistance genes. Cholera toxin B (ctxB) gene was amplified followed by sequencing.

**Results:** All the *V. cholerae* O1 isolates were El Tor Ogawa and showed the presence of the core toxin region representing the genome of the filamentous bacteriophage CTXφ. The complete cassette of virulence genes was seen in 48% of the isolates which was the predominant genotype. All the isolates possessed amino acid sequences identical to the El Tor ctxB subunit of genotype 3. sulII gene was detected in 68% of the isolates, dfrA1 in 88%, strB in 48% and SXT gene was detected in 36% of the isolates.

**Conclusion:** Toxigenic *V. cholerae* O1 El Tor Ogawa strains of ctxB genotype 3 carrying a large pool of virulence genes are prevailing in Assam. Presence of a transmissible genetic element SXT in 36% of the strains is of major concern as it indicates the emergence of multiple drug resistance among the *V. cholerae* isolates.

**Keywords:** *Vibrio cholerae* O1; Cholera toxin; Virulence; Genotype; Drug resistance

INTRODUCTION

Cholera still remains a global threat to public health in the developing countries where access to safe water and adequate sanitation cannot be assured for all. There are 2.9 million cases of cholera with 95,000 deaths occurring annually in endemic countries between 2008-2012. India is a country with more than 100,000 cases occurring annually (1). However, the actual global burden of cholera is not known as the vast majority of cases are not reported. WHO estimates that only 5-10% of the cases occurring annually are officially reported (2).

The causative agent of cholera, *Vibrio cholerae* is antigenically diverse organism and based on antigenic diversity of their outer membrane lipopolysaccharides, 206 serogroups (O1-O206) have been identified (3, 4). *V. cholerae* serogroup O1 is further classified into two biotypes, classical and El Tor, and two major serotypes, Ogawa and Inaba (5). Global...
replacement of *V. cholerae* classical biotype by El Tor biotype, emergence of O139 serogroup and rapid spread of antibiotic resistant strains indicate the continuous evolution in *V. cholerae*. The altered variant of El Tor strains containing the classical type of *ctxB* currently dominates globally.

Pathogenesis of *V. cholerae* is a complex process that involves coordinated expression of several virulence-associated genes to cause disease. The complete virulence profile of *V. cholerae*, including direct and indirect expression of genes involved in its survival and stress adaptation in the host are yet to be fully explored. The two major virulence factors that are associated with the generation of the symptoms of cholera are the cholera toxin (CT) responsible for the production of profuse rice-watery diarrhea and the toxin-coregulated pilus (*tcp*), a type IV pilus that mediates adherence, microcolony formation and intestinal colonization (6). Interestingly, the production of these virulence factors (CT and tcp) is strongly influenced by environmental conditions. Cholera toxin (CT), and toxin co-regulated pilus (TCP) are part of the genome of two horizontally acquired Mobile Genetic Elements (MGEs), CTXΦ, and Vibrio pathogenicity island 1 (VPI-1), respectively (7).

Apart from CT, the pathogenesis of cholera also relies on the synergistic action of a number of other genes like *tcp, ace* (accessory cholera enterotoxin), *zot* (zonula occludens toxin) and *toxR* the regulatory gene for CT production. Furthermore, the hemolysin of *V. cholerae* (*hly*), is extracellular membrane damaging proteins plays important role in manifestation of cholera (8). *ompW* is the species-specific gene and *rfbO1* genes confirm O1 serogroup of the isolates (9).

Emergence of new strains in any species of bacterial population have always been challenging. *V. cholerae* being no different from other bacteria, persists around the globe and undergo evolution over time. Its dual characteristic feature of being able to be sustained in the environmental (less infectious) and infectious state, and changing or acquiring various virulence genes or mobile genetic elements makes it difficult to understand the pathogenesis of *V. cholerae*. Increased poverty, overall reduction in water quality, excessive misuse of antibiotics and climatic changes are encouraging further genetic exchange of virulence and antibiotic resistance (6).

Assam is a state which is fed by the very dynamic and unstable river Brahmaputra and its tributaries causing frequent floods during monsoon due to incessant rainfall. Increased rainfall is associated with increased risk of cholera because during the flood, sewage water contaminates surface and groundwater that individuals rely on for drinking, bathing, and washing clothes which is a natural fauna of cholera in Assam. Apart from that, earlier studies have reported that the spatiotemporal serotype shifts and genetic diversity of *V. cholerae* strains between epidemics and pandemics may be due to climatic variations influencing the switching of virulence factors (10-12).

Globally, increased cases of dynamic *V. cholerae* strains, their serological switching and disease occurrence with respect to climate change (warm climate- more, cool climate- less) have attracted the attention of the public health sector (13).

Also, Assam is a state with highly diverse population in terms of ethnicity, religion and language. Of the 220 separate ethnic groups of North east India (NER), more than 30 of them are in Assam, which has over 70% of the North east region’s population. There has been a consistent flow of migration in this region because of employment opportunities in tea gardens, availability of cultivable land and other related factors. Assam also has interlinked borders with other states of NER as well as other countries like Bhutan and Bangladesh and may have different genotypic divergence in their virulence profile and antibiotic resistance pattern, the study of which may help to formulate the prevention and control strategy of cholera in the state.

To the best of our knowledge, no published literature has been found describing the virulence and antibiotic resistance gene profile of *V. cholerae* in Assam. As per Integrated Disease Surveillance project (IDSP) data, 48 outbreaks of cholera have been reported from various districts of Assam during the period 2012 to 2015. No data related to the molecular characterization of the isolates from these outbreaks have been published so far. The emergence of antimicrobial resistance is also a global phenomenon. Multiple drug-resistant *V. cholerae* strains was reported from this region earlier also (14).

In this context, the present study was undertaken to characterize the *V. cholerae* strains based on distribution of virulence associated genes and antibiotic resistance genes among *V. cholerae* strains in Assam and to compare the virulence profile of the strains with that of the strains circulating in other regions of India.
MATERIALS AND METHODS

Study design. The study was carried out after obtaining approval from the Institutional Ethics Committee. Acute watery diarrhea (AWD) cases referred to the hospital from various districts of Assam were included in the study after taking written consent. Twenty-five Vibrio cholerae O1 strains El Tor isolated by standard bacteriological methods during a period of two and half years were included in the study (15, 16). For further confirmation, serotyping was done using antisera obtained from DENKA, SEIKEN CO LIMITED, Tokyo, Japan.

Multiplex PCR for detection of biotypes and virulence genes of V. cholera. V. cholerae strains were subcultured on Luria-Bertani agar, suspended in sterile deionized water to a turbidity of 2.0 McFarland standard and then boiled for 10 minutes. Two sets of multiplex PCR assay were performed to detect the presence of diverse gene traits. The first multiplex PCR detected tcpA (El Tor), ctxA, O1rfb, rtxC, ompU, hlyA, zot, and ompW genes. The second set of multiplex PCR detected tcpA (Classical), ace, toxR genes. The primers are listed in Table 1 (9, 17-21).

The reaction mixture consisted of dNTPs (200 µM), MgCl₂ (1.5 mM), Taq polymerase (1U), primers specific for target gene (2 µl each), template DNA (100 ng), and milli-Q water. The thermal cycling condition for both the multiplex PCR was pre-incubation at 94°C for 2 min followed by denaturation for 1 min at 94°C, annealing for 1 min at 59°C, extension for 2 min at 72°C and incubation at 72°C for 10 min for a final extension.

After the completion of 30 cycles of reactions, 10 µl of each of the PCR products were mixed individually with 2 µl of gel loading buffer. Electrophoresis was done on 2% agarose along with 100bp ladder as a molecular marker and stained with 0.5 Mg/ml of ethidium bromide and the bands were observed using a UV transilluminator (Fig. 1A and B). Genotyping was done based on the presence or absence of the virulence genes (Table 2).

Multiplex PCR procedure was standardized using reference strains of Gastrointestinal Tract Pathogen Repository (GTPR) work V. cholerae O1 Ogawa (GTPR ID 413), V. cholerae O1 Inaba (GTPR ID 842), V. cholerae O139 (GTPR ID 1144), V. cholerae classical (MTCC 3904) and V. cholerae O139 (MTCC 3906) obtained from National Institute of Cholera and Enteric Diseases (NICED), Kolkata and Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India (MTCC) respectively.

Multiplex PCR for detection of antibiotic resistance genes of V. cholerae O1. Another Multiplex PCR assay was performed to detect antibiotic resistance genes for sulII (encoding sulfamethoxazole resistance), dfrA1 (O1-specific trimethoprim resistance), strB (Streptomycin B resistance), SXT (novel transmissible genetic element which contains the genes encoding resistance to these antibiotics), dfrl8 (O139-specific trimethoprim resistance) (Fig. 1B). The primers are listed in Table 1 (22-25).

Sequencing of ctxB gene. To determine the genotype of cholera toxin, Cholera toxin B (ctxB) gene was amplified from the isolates using the ctxB F GCCG-GGTTGTGGAATGCTCAG and ctxB R CAT-GCGATTGCCGAATTAGTGGC primers as described earlier (17). Purification of amplified product was performed by Exonuclease I-SAP (Shrimp Alkaline phosphatase) from Thermo Scientific, USA. Eluate was used as a purified gene product for sequencing which was performed for both the strands using the above-mentioned primers and BigDye® Terminator Cycle Sequencing Kit, Version 3.1 (Applied Biosystems, Foster City, USA). The sequencing reactions were analyzed on ABI 3500 Genetic Analyzer (Applied Biosystems® Foster City, USA). The consensus sequences were prepared using BioEdit sequence alignment editor (version 7.2.5) and searched against the GenBank database of the National Center for Biotechnology Information (NCBI) using the BLASTN algorithm (http://blast.ncbi.nlm.nih.gov/). The sequences obtained here were deposited in GenBank and the accession numbers are MK016537, MK016538, MK016539, MK029003, MK029004, MK29005.

RESULTS

All the strains tested were positive for the species-specific gene ompW and serogroup-specific O1rfb, thus confirming that all the isolates were V. cholerae O1. Detection of biotype-specific tcpA gene and rtxC gene revealed that, all the isolates belonged to biotype El Tor. Serotyping revealed that, all the isolates belonged to serotype Ogawa.
Table 1. List of primers used for detection of biotypes, virulence, and antibiotic resistance genes

| Serial No. | Target | Nucleotide sequence (5’→3’) | Amplicon Size (bp) | Reference |
|------------|--------|-----------------------------|--------------------|-----------|
| **First set of PCR primers** | | | | |
| 1 | tcp A (F), El Tor | GAAGAAGTTTGTAAAGAGAAGACAC | 472 | Keasler & Hall (1993) (18) |
| | tcp A (R), El Tor | GAAGACCTTCTTTACGTGATT | | |
| | ompU (F) | CCAAAACCGTGACAAACG | 655 | Kumar et al. (2009) (19) |
| | ompU (R) | TTCCATCGGTAAGAAGCG | | |
| 3 | rfb O1 (F) | CTATGGTCGTCCAGTTGTT | 638 | Goel et al. (2007) (17) |
| | rfb O1 (R) | CCCGATGACATACTTGACAG | | |
| 4 | rtxC (F) | CGACGAGAGATGAGCGAC | 265 | Chow et al. (2001) (20) |
| | rtxC (R) | CATCGTCGTTATGGTTGCG | | |
| 5 | cts A (F) | TCTACACTTTGAGCCCTATTGCC | 301 | Keasler & Hall (1993) (18) |
| | cts A (R) | TCTATCTCTGTAAGCCCTATTGCC | | |
| | zot (F) | TCCGTAAAGATGAGCCGTTT | 947 | Singh et al. (2001) (21) |
| | zot (R) | AACCCGAGCTACTTCTACCCA | | |
| 7 | hlyA (F) | GAGCGGTCATTCATCTGAAT | 480 | Kumar et al. (2009) (19) |
| | hlyA (R) | TCTACGGAATCCTACGTTTAA | | |
| 8 | ompW (F) | CACGAAAGTGCATTTTATTTG | 304 | Nandi et al. (2000) (9) |
| | ompW (R) | GGGTTCGATCAATGCGTAAAG | | |
| **Second set of PCR primers** | | | | |
| 9 | tcp A (F), Classical | CACGATAAGAAAAAACCGTGCAAGAG | 618 | Keasler & Hall (1993) (18) |
| | tcp A (R), Classical | ACCAAATGCAACGCGGATGACG | | |
| 10 | ctsB (F) | GCGGTTGTTGGGATGATCCTCAAG | 536 | Goel et al. (2007) (17) |
| | ctsB (R) | CATCGGATGTCGCACATATGACTGCG | | |
| 11 | ace (F) | TAAAGATTGTCCTATAGTGACACCC | 309 | Kumar et al. (2009) (19) |
| | ace (R) | CGTGATGATATGAGATATCTAGG | | |
| 12 | toxR (F) | CCTCTCAGATCCATCAAGAC | 779 | Singh et al. (2001) (21) |
| | toxR (R) | AGGTTTAGCAACGATGCGTAAG | | |
| **Primers used for detection of antibiotic resistance genes** | | | | |
| 1 | sull (F) | TGGCCGGATGAAGTCGCTCC | 626 | Hochhut et al. (2001) (22) |
| | sull (R) | AGGGGCCAGATGTAGTGAC | | |
| 2 | dfrA1 (F) | CAAGTTTATCATGCAAAATGGAAGCTGAT | 278 | Falbo et al. (1999) (23) |
| | dfrA1 (R) | ACCCTTTGTGCGAGTTTGTAG | | |
| 3 | strB (F) | CCGGATAGCTATAGCCGTGTT | 515 | Ramachandran et al. (2007) (24) |
| | strB (R) | CGACTACCGGCGACGGAAAT | | |
| 4 | SXT (F) | ATGGCCGTATCTAGCTAGCCTGC | 1035 | Bhanumathi et al. (2003) (25) |
| | SXT (R) | GCGAAAGTCATGCAGAACC | | |
| 5 | dfr18 (F) | ACTGCGGGTTGGCAGATGTCG | 389 | Hochhut et al. (2001) (22) |
| | Dfr 18 (R) | GGGTAAAGCAGCCTGTCATGG | | |

Among the virulence genes, ctsA gene was present in 88%, ompU in 64%, zot in 64%, hlyA in 84%, ace in 92% and toxR in 100% of the isolates. Based on the virulence genes analyzed, five genotypes of V. cholerae O1 were identified. Genotyping revealed, genotype tcpA (El)+ ctsA+ ompU+ rtxC+ ompW+ zot+ O1rfrb+ hlyA+ ctsB+ ace+ toxR+ i.e. complete cassette of virulence genes was identified in 48% of the V. cholerae strains and was the most predominant genotype (Table 2).

Detection of antibiotic resistance genes. sull was detected in 68% indicating resistance to sulphamethoxazole, strB in 48% indicating streptomycin B
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Fig. 1. A-Multiplex PCR 1- Lane 1, 6 & 9 (Genotype 1) showing complete cassette of virulence genes tcpA El Tor (472 bp), rtxC (265 bp), ompU (655 bp), hly A (480 bp), zot (947 bp); B- ace (309 bp), toxR (779 bp); Lane 2 (Genotype 2): Absence of zot; Lane 3, 7 & 8 (Genotype 3): Absence of hlyA and zot; Lane 4: 100 bp DNA ladder; Lane 5 (Genotype 4): Absence of ctxA; B-Multiplex PCR 2- Lane 1, 3, 4, 6, 7, 9-11 (Genotype 1): ace (309 bp), tox R (779 bp); Lane 2 & 8 (Genotype 5): Absence of ace; Lane 5: 100 bp DNA ladder.

Table 2. Representative genotypes of Vibrio cholerae O1 strains

| Strain | District                        | Genotype                                                                 |
|--------|--------------------------------|--------------------------------------------------------------------------|
| 12     | Kamrup Metro and Rural         | 1. tcpA (El) + ctxA + ompU + rtxC + ompW + zot + O1rfb + hlyA + ctxB + ace + toxR+ |
| 5      | Darrang                        | 2. tcpA (El) + ctxA + ompU + rtxC + ompW + zot + O1rfb + hlyA + ctxB + ace + toxR+ |
| 3      | Barpeta                        | 3. tcpA (El) + ctxA + ompU + rtxC + zot - ompW + O1rf + hlyA + ctxB + ace + toxR+ |
| 3      | Barpeta & Nalbari              | 4. tcpA (El) + ctxA - ompU + rtxC + ctxB + ompW + zot + O1rfb + hlyA + ctxB + ace + toxR+ |
| 2      | Morigaon and Golaghat          | 5. tcpA (El) + ctxA + ompU + rtxC + ompW + zot + O1rfb + hlyA + ctxB + ace - toxR+ |

aData are numbers of strains showing the same genotype
bAbbreviations: El-El Tor

d resistance, dfrA1 in 88% indicating trimethoprim resistance. Detection of SXT element in 36% of the isolates indicates that a considerable proportion of the isolates possess the transmissible genetic element which contains the genes encoding resistance to multiple antibiotics.

**Genotyping based on ctxB gene.** The sequences of ctxB gene of reference strains Vibrio cholerae O1 El Tor N16961 (Accession No. NC_002505) and Vibrio cholerae OI classical 569B (Accession No. U25679), El Tor, Australia (2463, 1988) were retrieved from GenBank. The deduced amino acid sequences of the ctxB gene from all strains were aligned using CLUSTAL W.

The deduced amino acid sequence alignment of partial ctxB subunit of Vibrio cholerae O1 El Tor isolated in the present study against reference strains revealed that, the isolates of present study possessed amino acid sequences identical to the El Tor type of CT-B subunit, which was 100% identical to the amino acid sequence of El Tor reference strain N16961 of genotype 3 by having aspartate at position 28, tyrosine at position 39, phenylalanine at position 46 and isoleucine at position 68 (Table 3).

**DISCUSSION**

The *V. cholerae* O1 El Tor isolates of the present study possessed different virulence genes like ctxA, O1rfb, rtxC, ompU, hlyA, zot, ompW, ace, toxR that indicates some toxigenic strain of *V. cholerae* are circulating in Assam. Cholera toxin (CT) is the most important epidemic marker among various toxins produced by *V. cholerae*, which is encoded by a mobile element, the genome of a filamentous CTX bacteriophage (26). Three strains of *V. cholerae* were negative for ctxA after repeated PCR. These findings may suggest that some *V. cholerae* strains can cause
illness in the absence of the cholera toxin. The absence of the ctxA gene may also be due to the ctxP prophage genome being missing or disrupted by mutations (27). However, the study could not exclude the possibility of strain diversity which probably could not be detected by the primers used in the study. All the ctxA negative strains were positive for accessory cholera toxin (ace), zonula occludens toxin (zot) and haemolysin A (hlyA) genes. This finding is similar to Alishahi et al. (2013) and Abana et al. (2019) (27, 28). All isolates were positive for tcpA El Tor which is similar to the findings of Abana et al. (2019) and Zaw et al. (2019) (27, 29).

In the present study 8 (32%) isolates possessed the ctx gene without the zot gene as well as 3 (8%) of the isolates possessed zot gene without the ctx gene, which is similar to a study conducted by Akoachere et al. (2014) (30). This may suggest that occurrence of zot gene may be independent of the ctx gene.

The screening for virulence genes revealed five different patterns or genotypes thus revealing the diversity among the strains even though all of them were clinical isolates pertaining to different districts of Assam. The predominant genotype included all the virulence genes investigated in the study. Determination of the genotypes based on the presence or absence of specific virulence genes only through in vitro test may not always indicate the severity of the strain as the expression of them in individuals may vary. However, the analysis of the virulence genes showed that the positive rates for the major virulence genes were mostly seen among the strains from Kamrup rural district which had large outbreak of cholera. A comparison of the virulence gene profile of V. cholerae isolates from different regions of India is presented in Table 4.

Sequencing of the partial ctxB gene revealed that the cholera outbreaks in certain districts of Assam were due to V. cholerae O1 El Tor carrying the ctxB gene of genotype 3. However, Borkakoty et al. (2012) reported circulation of V. cholerae O1 El Tor variant carrying the classical ctxBC gene in upper Assam (31). Chatterjee et al. (2009) and Goel et al. (2010) also reported the presence of V. cholerae O1 El Tor variant carrying the classical ctxBC gene in eastern and southern India respectively (32, 33).

Multiple antibiotic resistances among V. cholerae have emerged as a major problem worldwide. In India, there is a progressively increasing trend of antibiotic resistance towards common antibiotics like tetracycline and fluoroquinolones (14, 34-36). Resistance to these antibiotics could be due to the extensive use of these antibiotics for the treatment of other infectious diseases. The re-emergence of the cholera epidemic and the evolution of multidrug-resistant V. cholerae over the last decade, particularly in Asian countries, pose a great threat to the clinical diagnosis and treatment of cholera. The utmost concern is the acquisition of the SXT element by 36% of the isolates in the study. Similar to other bacteria, the spread of antibiotic resistance in V. cholerae is also facilitated by horizontal gene transfer via self-transmissible mobile genetic elements like SXT. Although identifying the serogroup and serotype of V. cholerae isolates is not necessary for treatment of cholera, this information may be of epidemiologic and public health importance. However, earlier studies have reported that, multiple drug resistance was more prevalent among the V. cholerae O1 El Tor Ogawa serotypes (31, 34). Hence, active surveillance is required for continuous monitoring of the serotype shift and emergence of antibiotic resistance.
Table 4: Biotype and genotypic characteristics of V. cholerae in different regions of India

| Region         | Biotype | Genotype |
|----------------|---------|----------|
| Andhra Pradesh| O1 El Tor| O1 El Tor|
| Maharashtra   | O1 El Tor| O1 El Tor|
| Karnataka     | O1 El Tor| O1 El Tor|
| India          | O1 El Tor| O1 El Tor|

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In conclusion, this study showed circulation of multidrug-resistant toxigenic V. cholerae O1 strains in Assam, with a trend towards increasing resistance to multiple antibiotics. The genotypic and phenotypic characteristics of these strains were diverse, indicating the need for further studies to understand the evolution and transmission dynamics of V. cholerae in Assam and other regions of India.
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