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Emergence of classical *ctxB* genotype 1 and tetracycline resistant strains of *Vibrio cholerae* O1 El Tor in Assam, India

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ABSTRACT

Cholera epidemics with moderately high case fatality rates in Assam, northeast India were investigated in 2007, 2008 and 2010. Based on mismatch amplification mutation assay PCR for detection of *ctxB* allele, 40 isolates of *Vibrio cholerae* O1 El Tor collected from the epidemics were found to harbour the classical *ctxB* gene allele of cholera toxin (CT). DNA sequencing of *ctxB* gene confirmed the isolates to be genotype 1 of *ctxB*. Antimicrobial susceptibility tests reveal that 100% of the isolates were resistant to trimethoprim and 40% were resistant to tetracycline. The recent *V. cholerae* O1 strains circulating in Assam, India are due to the El Tor variant carrying classical type CT. Emergence of tetracycline and trimethoprim resistant strains necessitates the review of antibiotic use for severe cholera.

1. Introduction

Cholera caused by *Vibrio cholerae* O1 or O139, is an acute diarrheal disease that can kill within hours if left untreated.[1] Although cholera is endemic in many developing countries, it has re-emerged as an important public health problem in a number of countries with inadequate sanitation,[2] made worse by natural calamities like floods and earthquakes. There have been reports of severe cholera and increasing case fatality rates (CFR) due to cholera in recent times,[2] as in the recent outbreak in 2010 in Haiti and the Dominican Republic where around 1882 deaths due to cholera were reported with an in-hospital CFR of 3.4%.[3] Outbreaks of cholera from different regions of the world have reported the emergence of strains of *V. cholerae* O1 with El Tor variant carrying classical *ctxB* allele (*ctxB*^{C}) of cholera toxin (CT).[4–9] *Vibrio cholerae* O1 with typical El Tor phenotypes (resistant to 50 units of polymyxin B, and positive for chicken erythrocyte agglutination [CEA] and Voges-Proskauer [VP] test) but carrying *ctxB*^{C} are designated as El Tor variant.[8]

Vibrio cholerae O1 is classified into two biotypes, classical and El Tor, and into three serotypes, Ogawa, Inaba and Hikojima.[10] The classical biotype was responsible for the six pandemics starting from the early nineteenth century, while the seventh pandemic of cholera, which has been raging on since 1961, is caused by the El Tor biotype that has spread worldwide including the last bastions of developing countries.[10] The El Tor strains are more adaptable and resilient in the environment, have more asymptomatic carriers than the classical counterpart and cause higher infection to case ratio.[11] It has been observed that the classical biotype usually produces more severe illness.[10] Cholera toxin, the key toxin of *V. cholerae* O1 and O139, is made-up of five identical B subunits coded by the *ctxB* gene and a single A subunit coded by the *ctxA* gene.[10] Based on the *ctxB* gene, CT of classical and El Tor biotype can be classified into three genotypes: 1, 2, and 3. Genotype 1 is found in the classical biotype worldwide and in the US gulf coast, genotype 2 is found in strains of El Tor from

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Australia whereas El Tor biotype of the seventh pandemic belongs to genotype 3.[12] Isolates of *V. cholerae* O1 El Tor biotype that belong to classical biotype ctxB (genotype 1) differ from genotype 3 by two nucleotides at positions 115 (codon 39) and 203 (codon 68) that code for histidine at codon 39 and threonine at codon 68. Genotype 2 has one nucleotide difference at position 138 (codon 46) from genotype 1, corresponding to an amino acid substitution from phenylalanine to leucine.[4,13]

The high CFR associated with recent circulating strains augmented by multiple antibiotic resistances could create a serious problem for public health in the near future. *Vibrio cholerae* has remained sensitive to most antibiotics for a long period, however, the scenario in recent years has changed and today *V. cholerae* strains isolated worldwide are resistant to multiple antibiotics.[14] Though oral rehydration treatment is the primary therapy in case of cholera, antimicrobials are essential at times to reduce the volume of stool and shorten the duration of the disease.[14] Keeping in mind the increasing public health threat for *V. cholerae*, the present study investigated epidemics of cholera in Assam in northeast India from four different outbreaks in tea garden communities in 2007, 2008 and 2010. Assam, with a population of over 31 million, faces two to three devastating floods every year and sporadic outbreaks of acute diarrheal disease is an annual occurrence. This study was conducted to evaluate the *V. cholerae* strains for the presence of classical-like *ctxB* and the antimicrobial susceptibility pattern of the strains during the epidemics.

2. Materials and methods

Investigations of outbreaks of acute watery diarrhea in the tea garden communities of Assam in the districts of Golaghat and Jorhat in 2007, Sibsagar in 2008 and Darrang in 2010 were conducted by the Regional Medical Research Centre North East Region, (Indian Council of Medical Research) Dibrugarh. Representative samples from the outbreaks were collected for investigation and analysis.

2.1. Bacterial strains and biochemical and serological characterization

In brief, rectal swabs transported in Cary-Blair medium (HiMedia, Mumbai, India) were enriched in alkaline peptone (pH 8.6) water for 5–8 hours and sub-cultured in thiosulphate citrate bile sucrose agar (TCBS). Typical sucrose fermenting yellow colonies in TCBS were further subjected to conventional biochemical tests for confirmation of *V. cholerae* as per standard protocol.[15] In this study 40 representative clinical isolates of *V. cholerae* O1 El Tor from all four outbreaks were subjected to further investigation by both conventional and molecular tests. All strains were biotyped by using standard procedures including susceptibility test to 50 units of polymyxin B, CEA, haemodigestion of blood agar and the VP test using strains 569B and N16961 as the classical and El Tor reference strains, respectively.[8,10,15] All strains were subjected to serogrouping and serotyping by serogroup specific O1 polyvalent antiserum and serotype specific antiserum for Ogawa and Inaba strains, respectively (BD, Sparks, USA).

2.2. Antimicrobial susceptibility testing

All isolates of *V. cholerae* O1 El Tor underwent an antimicrobial susceptibility test (AST) using 0.5 McFarland standard inoculums in sterile saline and tested in Mueller-Hinton agar (MHA) medium (HiMedia, Mumbai, India) plates and incubated at 35 °C. The ASTs were performed according to Clinical and Laboratory Standard Institute (CLSI) guidelines,[16] with commercial antibiotic discs for ampicillin10 μg, tetracycline 30 μg, trimethoprim 5 μg, ciprofloxacin 5 μg, ceftriaxone 30 μg and amikacin 30 μg (HiMedia). Zone size inhibitions were measured for individual antimicrobial agents according to CLSI guidelines. Susceptibility tests for polymyxin B 50 units and 300 units in nutrient agar (HiMedia) were carried out separately by standard methods.[15] *Escherichia coli* ATCC 25922 was used as the quality control strain for disc diffusion.

2.3. Detection of ctxB allele and DNA sequencing

Overnight cultures of all *V. cholerae* isolates grown in nutrient agar were used for nucleic acid extraction. In brief, 1.5 ml microcentrifuge tubes were loaded with 200 μl of distilled water. Three to four average size colonies from nutrient agar were added to microcentrifuge tubes with distilled water and mixed by vortexing. The tubes were put in a water bath at 100 °C for 20 min and snap-chilled in ice for 5 min followed by centrifugation at 10 000 g for 2 min. Supernatants were used as the genomic DNA templates.

Mismatch amplification mutation assay PCR (MAMA-PCR) was performed based on the method described by Moriata et al.[13] A common forward primer was used for the classical and El Tor alleles, Fp-Com (5′-ACTATCTTCA GCATATGCAC ATGG-3′) and 2 allele-specific primers Rp-cla (5′-CCTGGTACTT CTACTTGAAA CG-3′) and Rp-elt (5′-CCTGGTACTT CTACTTGAAA CA-3′), were used for classical and El Tor biotypes, respectively. Two sets of PCR reaction were set-up using the specific reverse primer for each allele in a different set. Each PCR was carried out in 20 μl final reaction volume using 10 μl of 2x PCR master mix (Promega, Madison, USA), 1 μl each forward and reverse primers (0.5 μM), 2 μl of bacterial genomic DNA template and final volume was adjusted with nuclease free water. Each PCR was performed with an initial denaturation at 96 °C for 3 min followed by 30 cycles of denaturation (96 °C for 10 s), annealing (55 °C for 10 s) and extension (72 °C for 30 s) and final extension at 72 °C for 2 min. The amplified fragments were detected by agarose gel electrophoresis after staining with ethidium bromide. No template control and reference strains N16961 (El Tor) and 569B (classical) were run in every PCR reaction. The partial *ctxB* gene of all 40 *V. cholerae* isolates were sequenced. In brief, PCR products were purified with High Pure PCR Product purification kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Purified PCR products were cycle sequenced using the ABI Big Dye Terminator