Research Article

Molecular Diagnosis of a Diarrheal Outbreak in a Hospital Ward

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Abstract

*Vibrio cholerae*, the causative agent of Asiatic cholera, is a gram-negative motile bacterial species acquired via oral ingestion of contaminated food or water sources. It is one of the major epidemic causing agents in India. Almost every developing country is now facing either a cholera outbreak or the threat of an epidemic, which along with serious public health problems severely affects the economy. 31 stool samples were collected on a diarrheal outbreak from Kilpauk Medical College and 20 water samples in around the hospital. These samples were processed by both conventional methods (enrichment followed by biochemical test) and direct DNA Isolation (by CTAB method) from the samples for PCR. Primers specific for the Cholera toxin gene (*ctx*) was used to identify the presence of virulent *V. cholerae*. The specificity & sensitivity of the primers used were already proven. All the samples were positive for the presence of *Vibrio* spp. by conventional methods but only 21 stool and 18 water samples showed positive for *ctx* gene. The result justifies the reason for outbreak is due to contaminated water. Also it shows that direct PCR method is more reliable than the conventional methods in explaining the toxigenic and the epidemic nature of the organism which undergoes a dormant stage alive but non culturable forms or alive without the toxigenic property. To study the molecular variation we sequenced the *ctx* genes which were matched against standard strain.

Keywords: *Vibrio cholera*; Cholera; CTX; PCR; Contaminated water; outbreak; Epidemic

INTRODUCTION

Cholera continues to be one of the major threats in India characterized by a severe watery diarrhea caused by toxigenic *Vibrio cholerae*, produces an enterotoxin (cholera toxin) by colonizing themselves to the small intestine (Norris, 1974; Rabbani and Greenough, 1990; WHO Scientific Working Group, 1980). In human volunteer studies, the infectious dose was determined to be fairly high, and varied depending on the inocula conditions (ranging from 106 to 1011 colony-forming units) (Bennish, 1994). This high dose is probably needed because of the acid sensitivity of *V. cholerae* cells, which are exposed to low pH in the gastric compartment (Cash et al., 1974). *V. cholerae* has been shown to survive for contradictory time periods in environmental waters (Feachem et al., 1981). The toxigenic O1 strains of *Vibrio cholerae* have been shown to survive in aquatic environments for years, possibly residing in a biofilm. Outbreaks of cholera cause deaths estimated at 120,000 annually worldwide and many more cases each year, of which the vast majority occurs in children. Almost every developing country is now facing either a cholera outbreak or the threat of an epidemic, where access to the safe drinking water and adequate sanitation was not assured.
A distinctive epidemiological feature of cholera is its appearance in a regular seasonal pattern in areas of endemic infection often starting in several distinct foci simultaneously, indicating a possible role of environmental factors in triggering the epidemic process (Glass et al., 1982; Kaper et al., 1985). The first of seven major cholera pandemics (widespread epidemics) began in 1817, when cholera spread from its endemic focus in India (Barua, 1992). V. cholerae is primarily an inhabitant of the aquatic environment, so water plays an important role in the transmission and epidemiology of cholera.

Toxigenic V. cholerae strains are lysogens of a filamentous phage (ctx U), which encodes cholera toxin (CT), the major virulence factor of the organism (Islam et al., 1994; Faruque et al., 1998). CT is a potent protein exotoxin that elicits a secretory response from epithelial cells, and this secretory response is the principal basis of clinical cholera (Sack et al., 2004). Although the clinical manifestations indicate the occurrence of the disease, yet rapid and accurate identification of V. cholerae in drinking water is required (Madico et al., 1996; Choopun et al., 2002). Culture methods require prolonged incubation and growth on selective media to reduce the number of non-specific organisms which are time consuming and laborious (Colwell et al., 1985; Barcina et al., 1997) and poses difficulty in identifying the viable and non culturable state of the organisms. Also these tests are not very reliable because it won’t give a clear picture of the toxigenicity. Therefore, nucleic acid-based techniques have emerged as powerful alternatives for fast and reliable identification.

The present study is to analyse the diarrheal outbreak occurred at the Ladies ward of Mental Hospital using nucleic acid-based techniques, to know the etiological factor of the disease and its source. 31 Stool samples were obtained from Kilpauk Mental Hospital and diagnosed for V. cholerae along with 20 water samples collected in and around the hospital environment to know the source of the outbreak.

MATERIALS AND METHODS

Collection of samples

31 stool samples were collected from the patients of Kilpauk Mental Hospital. 20 water samples were collected from in and around the (within 5 Km radius) Kilpauk mental hospital were concentrated and processed for the direct DNA isolation.

Culture methods

Stool samples were plated directly on the thiosulfate-citrate-bile salt (TCBS) agar. Water samples were concentrated 0.45 µm pore size membrane filters, and enriched in alkaline peptone water (APW (1% peptone, 1% NaCl, pH 8.4–8.6)) for isolation of Vibrio spp. (Kaper et al., 1979). Bacterial colonies were then isolated on TCBS agar. The on TCBS were confirmed by biochemical tests as described previously (Alsina and Blanch, 1994) for Vibrio spp.

Concentration and DNA isolation

100 ml of water samples were concentrated for bacteria by passing through the 0.45 micron size membrane filter and eluted using 50 ml of UAPB buffer. Followed by the flocculation of samples by adding 1 ml of 0.1 M MgCl₂ and centrifuged at 5,000 rpm for 30 minutes. Pellets were collected in 1ml of mcellavaines buffer and processed for DNA.

DNA isolation was carried out with 300 µl of stool samples and concentrated water samples by CTAB method (Del Sel et al., 1989).

PCR

The sense and antisense primers specific for ctx A gene used in this study were 5’CTC AGA CGG GATTTG TTA GGC ACG 3’ and 5’TCT ATC TCT GTA GCC CCT ATT ACG 3’respectively (Kessler and Hall, 1993). DNA amplification was carried out in gene amplification PCR system in a reaction volume of 25µl. Each reaction contains: 2 X PCR master mixes 12.5µl (master mix contains DNA polymerase MgCl₂ and dNTPs). Sense and antisense primer 10 picomoles along with 2µl of DNA from processed clinical and water samples and the reaction volume was adjusted to 25µl using nuclease free water.

The cycling profile was as follows: initial denaturation at 95°C for 5 min, and 30 cycles of denaturation at 94°C for 1 min, annealing at 55.5°C for 2 mins, extension at 72°C for 3 mins and final extension at 72°C for 5 min. The PCR products were electrophoresed along with 100 bp ladder on 1.5% agarose gel in 1X TAE.
Gene sequencing

For sequencing analysis, PCR amplicons were gel excised and purified using a QIA quick gel purification kit (Qiagen Inc., Valencia, CA), and DNA sequencing was carried out using ABI 310 Genetic Analyser (Shankra Nethralaya, Chennai, India) using both the forward and reverse primers to confirm the results.

RESULTS AND DISCUSSION

31 samples were collected from Kilpauk Medical College patients to study the Cholera outbreak occurred there. All the samples showed the presence of Vibrio spp., in the culture method followed by biochemical methods and only 21 were positive in PCR for CTX gene showing the band at 301 base pair (Figure 1). Among 20 water samples 12 were PCR positive for the CTX gene (Figure 2). All the samples were positive for Vibrio cholera by conventional biochemical methods. The distribution of positive cases (PCR) by age was shown in the table (Table 1).

The nucleotide sequence of the CTX A PCR product were compared with other sequences at the GeneBank using BLAST and the result shows the PCR amplified sequence was coming under the CTX A subunit gene. The accession numbers of the forward and reverse sequences submitted in the Genbank were GU902273 and GU902274 respectively.

Cholera is continually posing a health threat to developing countries. Cholera is an epidemic and life-threatening diarrhea, and is transmitted generally by the faecal-oral route. Poor sanitation practices in highly populated areas harboring endemic toxigenic strains are the source of occasional outbreaks due to contamination of drinking water and/or improper food preparation. Contaminated water with free-living V. cholerae cells are probably the main origin of epidemics, followed to a lesser extent by contaminated food, especially seafood products like oysters, crabs, and shellfish (DePaola, 1981; Kaysner, 1994). The sudden outbreak of cholera in the ladies ward of the kilpauk mental hospital provided us an unique opportunity to access the role of contaminated water during the outbreak.

The percentile distribution of the outbreak also proportionally increases with the age which also relates the outbreak with the age factor. Mahalanabis et al., also studied the distribution of Vibrio cases and found that 71 % of adults (>15 years) were infected with O137 infection (Mahalanabis et al., 1994)

Although all the samples were positive for the presence of Vibrio spp. through conventional methods only 18 water samples and 21 stool samples were positive for the presence of ctx gene. It shows the inability of the conventional methods to prove the toxigenic property of the isolates. Also Increasing evidence seems to suggest that, during the inter-epidemic period; the O1 serogroup of V. cholerae reverts into a viable but non-culturale state (Colwell and Spira, 1992). The studies with water samples collected in and around the hospital also showed positive for the for ctx A subunit which stands the source for the outbreak. Ghosh et al. (1994) states that there is an increase in the numbers of toxigenic vibrios among the innocuous aquatic residents facilitated by means of genetic exchange with some potential to provide a reservoir to reinitiate an epidemic.

Nowadays, PCR has become an important and powerful tool for detection of pathogenic micro-organisms in the environment (Bej and Mahbubani, 1992; Koenraad et al., 1995; Catalan et al., 1997). Direct identification of pathogenic organisms without prior isolation and purification from complex substrates such as fecal material, sputum, and urine has been accomplished by PCR (van Eys et al., 1989) which proves the specificity, and sensitivity of the PCR analysis and it is also a rapid technique to identify the presence of toxin producing gene directly.

The production of CT by V. cholerae is important from the perspective of a serogroup acquiring the potential to cause epidemics. Cholera toxin gene (ctxAB) is acquired from the genome of a filamentous ctx bacteriophage. This has become particularly evident since the emergence of V. cholerae O139. A dynamic 4.5-kb core region, termed the virulence cassette (Trucksis et al., 1993), has been identified in toxigenic V. cholerae O1 and O139 but is not found in non-toxigenic strains. It is known to carry at least six genes, including ctxAB which encodes A & B sub unit of the CT. ctxAB gene which is specific among various toxin producing strains of V. cholerae showed that cholera toxin is most potent and serves as a marker for epidemic
Figure 1: Gel electrophoresis of PCR products of stool samples

Figure 2: Gel electrophoresis of PCR products of water samples
potential (Kaper et al., 1995). Our sequence studies shows the amplified region was coming under the A subunit of the ctx gene which indicates epidemic nature of the organism.

Table: 1 Distribution of cases by age

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Positive Cases</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;20</td>
<td>1/3</td>
<td>33.33</td>
</tr>
<tr>
<td>21 – 30</td>
<td>4/7</td>
<td>57.14</td>
</tr>
<tr>
<td>31 – 40</td>
<td>8/12</td>
<td>66.67</td>
</tr>
<tr>
<td>41 – 50</td>
<td>7/8</td>
<td>87.5</td>
</tr>
<tr>
<td>&gt;50</td>
<td>1/1</td>
<td>100</td>
</tr>
</tbody>
</table>

Topical studies have revealed that virulence genes or their homologs are discrete among environmental V.cholerae strains belonging to sundry serogroups, whereas previously it was considered that virulence genes are carried only by the clinical isolates (Colwell and Spira, 1992). Molecular characterization of the genes provide information about the ecology of V. cholerae, which is an autochthonous inhabitant of aquatic environment as well as pathogenic for humans.

Vibrio cholerae O1 and non-O1 can shift to a rugose form associated with the production of an exopolysaccharide which promotes cell aggregation. (Morris et al., 1993) This rugose form resists chlorine (even at levels exceeding 2mg/l) and other disinfectants in potable water and is likely to contribute for water born transmission of cholera (Rice et al., 1992). The majority water treatment protocols follow only chlorine treatment to eradicate the microbial contamination but these rugose forms escapes the treatment and goes on causing diseases and that’s why they possess endemic state in many areas of the developing countries.

It is very important to develop new treatment protocols to eradicate these Vibrio cholerae in the water. Also periodic screening for both the toxigenic as well as non toxigenic strains was required to keep the outbreak under check because the intestine is supposed to be the site where these non-toxigenic strains can be converted into toxigenic ones (Faruque et al., 1998).

Thus it is very mandatory to keep tracking the purity of water in regular intervals provided the intervals may be short enough to make sure that we should not give the pathogen to form an outbreak.

REFERENCES


