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Isolation of Vibrio cholerae from clinical and drinking water samples during Cholera Outbreak in Khairpur Sindh Pakistan.

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Abstract

Background: Cholera is a diarrheal disease that is caused by the bacterium Vibrio cholera (V. cholerae) that transmits through contaminated water, food, oral-faecal route and spread through poor sanitation system. This study aims to investigate the reports of clinical and drinking water samples during 2014 to 2016 cholera outbreak in Pakistan.

Methodology: This study was conducted in District Khairpur including 660 samples (360 from clinical and 300 from drinking water). All samples were enriched in Alkaline peptone water for 6 hours and then streaked on Thiosulfate-citrate-bile salts-sucrose (TCBS) agar, incubated at 35ºC for 24 hours. Moreover, microbiological, biochemical, serological techniques were used for the identification of V. cholerae and further identification was performed using PCR.

Results: Out of 360 clinical samples, 76(21.11%) were positive for V. cholerae. The species-specific outer membrane protein W precursor (ompW) gene was amplified and shown at the correct size (588 bp) through agarose gel electrophoresis. The serotyping revealed that the isolates belonged to serogroup Inaba, O1. Cholerae O1 strains shown typical El Tor phenotype similar to V. cholerae El Tor strain N16961 (PBR VP+) used as the reference strain in this study. All age groups were affected where the highest onset was seen among those aged 19 years and above. The culture of drinking water (n=300), observed negative on TCBS agar.

Conclusion: As far as our knowledge, this is the first time when the presence of V. cholerae El Tor peak has been reported for causing cholera outbreaks in Khairpur Sindh, Pakistan. However, these findings can be used for further investigations and recognizing control measures.

Keywords

Vibrio cholerae, Cholera, El Tor, Disease Outbreaks, Electrophoresis, Agar Gel, Drinking Water.

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Introduction

Cholera is a very serious form of human-caused diarrhea by Gram-negative, motility, bacterial filamentous, and coma-sized called V. cholerae[1]. More than 200 cholera serogroups, only serge O1 and O139 groups are primarily responsible for cholera. Cholera has infected South Asia for at least 1,000 years, besides it has caused seven epidemics throughout the world since 1817[2,3].

Serogroup O1 contains classical and El Tor biotypes (based on special chemical tests and phase sensitivity) and possess three different serotypes, Inaba Ogawa and Hikojima[4]. The classical biotype caused six cholera outbreaks from 1817 to 1923[4,5]. Later, the El Tor biotype replaced the classical biotype that created the seventh cholera epidemic in 1961, and since then, V. cholerae O1 El Tor is the world's leading cholera organism[6,7]. A few years later, serogroup O139 was replaced by O1 serogroup, and now it does not appear[8,9]. In recent years, cholera has become even more common in developing lands where sanitation is poor and safe drinking water is scarcely available. For example, the most recent outbreak was reported in Yemen on April 27, 2017, and the disease resulted in a total of 332,658 suspected cases of cholera and 1,759 deaths as of 2017[10].

Cholera may be caused by infected people and water reservoirs such as water and rivers[11]. Cholera infection is most common in drinking water when V. cholerae is found naturally or in the feces of an infected person. Transfers from person to person, even to health care workers during an epidemic, are documented[12,13]. V. cholerae pathogenicity is caused by a genetic code of proteins that are directly or indirectly involved in the bacteria virulence[14].

At the time of infection, V. cholerae also releases cholera toxin, a protein that causes diarrhea. In 2010, there was an increase in cholera cases due to the worst floods that threatened public health throughout Pakistan, with 164 confirmed cases in the laboratory, where cholera cases had previously been hardly reported[15]. Cholera remains a major health problem in Pakistan, with many cases every year, especially during the rainy season. Because of poor living conditions and sanitation practices, Pakistan is facing health risks, including gastroenteritis[16-18].

This study aims to investigate the reports of clinical and drinking water samples during 2014 to 2016 cholera outbreak in Pakistan. Our findings may also provide important insights into the outcome of V. cholerae O1 El Tor isolates[17-18].

Methodology

In this study, V. cholerae was isolated from the clinical and drinking water samples from various Khairpur hospitals, including both indoor and outpatient patients of Khairpur Medical College Teaching Hospital (Main and City branch) Khairpur and Pir Abdul Qadir Shah Jeelani Institute of Medical Sciences (PAQSJIMS) Gambat. The stool/rectal swabs were collected from the patients with the standard safety measures. The study was approved by the Ethical Review Committee of Natural Sciences Shah Abdul Latif University, Khairpur.

Table 1: Sampling Sources during this study for Isolation of V. cholera.

Source	Site	Total samples
Stool samples/rectal swabs	KMC Teaching Hospital Khairpur	320
Stool samples/rectal swabs	PAQSJIMS Gambat	40
Drinking water	Khairpur Municipal water supplies (30 wards)	120
Drinking water	Ground water Khairpur and Gambat	120
Ice Factory water	Khairpur city (10 factories)	60

All samples were then transferred to the Molecular Biology laboratory, Institute of Microbiology Shah Abdul Latif University Khairpur for enrichment in alkaline peptone water[19] (1% Bacto Peptone, 0.5% NaCl, 0.07% Na_2CO_3, 0.01% KNO_2, and pH 8.6) and incubated at 35ºC for 6 hours, then the enriched samples were streaked on thiosulphate citrate bile salt sucrose agar (TCBS, Oxiod) selective for V. cholerae and incubated aerobically at 35ºC for 24 hours. The next day the growth was observed for sucrose fermenting distinct yellow V. cholerae colonies. These colonies were Gram stained, sub-cultured on a non-selective medium (Nutrient agar, Oxiod), and incubated aerobically at 35ºC for 24 hours. Presumptive identification of V. cholerae was performed through a motility test using Hanging drop Technique[20] and string test[21]. Biotype characterization was performed for confirmation of V. cholerae using API 20E (Analytical profile index for Enterobacteriaceae; bio Merieux, Inc., Hazelwood, MO) as described by Patrick[22] as shown in figure 1. Isolates were tested for biotype using classical methods viz Voges-Proskauer (VP) test and Antibiotic sensitivity against Polymyxin B (Oxoid 300 U). V. cholerae classical is sensitive while El Tor is resistant to Polymyxin B. Sero-grouping was performed using specific polyvalent antisera against V. cholerae O1 and O139, and monovalent antisera specific to Inaba and Ogawa (Denka Seiken Co. LTP-JAPAN) using slide agglutination. V. cholerae O1 were preserved at −20ºC in glycerol for subsequent detection by species-specific PCR targeting ompW gene (588 bp)[15-23], using specific primers ompW forward and reverse as described[15-23].

DNA extraction was carried out by boiling method as described by Sepp and colleagues[24]. Well, isolated colonies from fresh culture were suspended in Tris-EDTA (TE buffer) and boiled for 10 minutes. After that, the tubes were placed on ice for 2 minutes, centrifuged at 13000 rpm for 10 minutes at 4ºC, and the supernatant was transferred to clean sterile 1.5 ml tubes and stored at -20ºC.

Figure 1: Identification of V. cholerae by Biotypic using API 20E (n=76)

Polymerase chain reaction (PCR)

Hot-start PCR was performed for amplification of ompW gene from indigenous V. cholerae strains and ATCC 14035 V. cholerae strain used as a positive control. The parameters were 1 cycle pre-denaturation at 95ºC for 5 minutes, 35 cycles denaturation at 95ºC for 1 minutes, annealing at 61ºC for 30 seconds, extension at 72ºC for 1 minutes and final extension at 72ºC for 10 minutes. A Hybrid thermal cycler was used for all reactions. The amplicons were stored at -20ºC[15].

Agarose gel electrophoresis

Agarose Gel Electrophoresis was performed as described in Shah et al[15]. The gel was observed by using the ABI Gel doc system and photographed. The amplified DNA products were visualized using 1% agarose gel electrophoresis under a UV transilluminator (BioRad USA).

Calculation of isolation rate of V. cholerae

The isolation rate was calculated using the following equation[24].

$$\text{Isolation rate (\%)} = \frac{\text{No. of samples positive}}{\text{No. of samples tested}} \times 100$$

Results

Isolation rate and phenotypic characterization of V. cholerae from clinical samples

The isolation rate of V. cholerae strains obtained from the clinical samples was 21.11%, where the isolation rate was 0 for all the drinking water samples (n=300) tested in this study. Clinical isolates of V. cholerae showed 24 hours as the optimum time for growth, and the optimum temperature was 35ºC. On TCBS agar, typical sucrose fermenting yellow 2- 4 mm shiny colonies appeared, whereas translucent, shiny colonies appeared on Nutrient agar.

The Gram staining showed typical Gram-negative curved rods and darting motility by hanging drop technique, and the string test was found positive in all the clinical isolates. Phenotypic characterization is shown in table 2. Bio typing using API 20 E exhibited a specified ID number (5347124) for V. cholerae (Figure 1). The biochemical profile was typically that of V. cholerae (Table 2) confirmed that the isolated strains were V. cholera.

Table 2: Phenotypic Identification of Indigenous V. cholerae Strains.

Test	Result
Growth on TCBS agar	Sucrose fermenting Yellow colonies 2-4 mm in diameter
Growth on Nutrients Agar	Shiny colonies 2-4 mm in diameter
Gram's stain	Gram-negative curved roads
Motility Test	Positive (Darting)
Oxidase Test	Positive
String Test	Positive
Indole Test	Positive
Methyl Red	Positive
Voges- Proskauer Test	Positive
Citrate test	Positive
Triple sugar Iron Test (TSI)	Negative No gas production
Trypton broth with 0% NaCl	Positive

Sero-grouping and bio-typing of indigenous V. cholerae

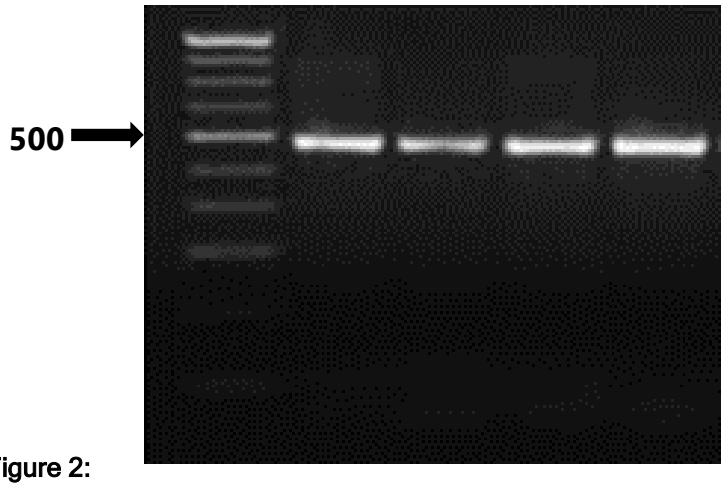
Sero-groping of the indigenous strains with polyvalent antisera showed that the V. cholerae belong to serogroup Inaba, O1. The biotype classification of the V. cholerae O1 strains revealed a typical El Tor phenotype similar to that of El Tor strain N16961 (PBR VP+). In our study, the isolated strains were Polymyxin B resistant, therefore, confirmed as El Tor biotype.

Genotypic characterization of indigenous V. cholera-DNA Extraction (n=76)

Genomic DNA from isolates was extracted as described in materials and methods. Agarose gel electrophoresis revealed the presence of genomic DNA in all the indigenous samples (not shown). All the samples showed a band approximately of similar size.

Amplification of ompW Gene from indigenous V. cholerae

The agarose gel electrophoresis revealed the successful amplification of own gene in all the clinical isolates at the correct position of 588 bp comparable to the ompW gene of V. cholerae ATCC14035 used as a positive control in this study (Figure 2).



From left to right
Lane. 1-DNA ladder 1000 bp
Lane. 2-(+ve) control ATCC 14035
Lane. 3-5-AMVC strains showing band of ompW virulent gene at 588 bp position

Figure 2: Agarose Gel Electrophoresis of Clinical V. cholerae from this study.

Discussion

The present study aimed to examine the phenotypic and genotypic characteristics of native V. cholerae. Cholerae isolated from clinical samples of gastrointestinal patients with drinking water to detect the proliferation of indigenous Cholera areas selected from Khairpur Sindh Pakistan. Out of the 360 clinical samples, 76(21.11%) V. cholerae isolates have been identified according to standard microbiological, biochemical, serological techniques and specific types of PCR targeted at ompW gene.

V. cholera isolated from this study belonged group O1 which is consistent to the findings of Jabeen et al[25]. Isolation of V. cholerae O1 has revealed that all 76 cases identified as normal El Tor are similar to the phenotype of the 7th epidemic El Tor N16961 (PBR VP+). There was male domination over women (n=348, 96.6%). Where the most affected age group was 19 years and older. Our data agree with the recently reported findings from Pakistan[26].

Although cholera often affects outside of gender or age, however, these factors may play a role in the incidence of cholera, which can affect the diagnosis. This, therefore, puts certain social groups at high risk of contracting and spreading the disease in a particular setting. As the men in the study area were more exposed to foreign activities they were the bread earning members in their families; as a result, the incidence rate was higher in this study group.

It is important to note here that no drinking water samples were found suitable for V. cholerae even in the high incident months, i.e. May to August (determined in this study) of cholera accompanied by reports of high outbreaks of cholera during the pre-rainy season of the year, with the second-highest number in the post-rainy season[27]. The adverse effects of the culture of water samples in our study may be due to the fact that V. cholerae may live in contaminated water for a long time in an inactive state and infect only in the human stomach[28,29]. This poses a threat to human health due to its no recovery from water as non-culturable status in water, or it may be simply a reaction to unfavorable conditions and can be counterproductive to heterotrophic invaders and bacteriophages by adopting this unique survival strategy of an unculturable state[30].

Conclusion

In the present study, of 360 clinical cell samples, 76 clinical isolates were identified as V. cholerae serogroup O1 Inaba, biotype El Tor. Genotypic mutations have shown the presence of virulence gene ompW in isolates. Additional studies are required to further investigate the mechanism of infection and factors promoting the overall prevalence of V. Cholerae, as it seems potentially invasive in the population of Khairpur.

Conflicts of Interest

The authors have declared that no competing interests exist.

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