Characterization of *Vibrio cholerae* from deep ground water in a cholera endemic area in Central India

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Abstract A total of 8 out of 11 deep ground water samples collected from different villages in Central India were found contaminated with *Vibrio cholerae* non O1, non O139. In a multiplex PCR, isolates were found positive for *ompW* gene but negative for *ctxAB* and *rfbO1* genes. However, isolates from two places were positive for *tcp* and *zot* genes, indicating their intestinal colonization and toxigenic potential. Antibiotic susceptibility studies revealed that all isolates were multidrug resistant. Although, none of the isolates was found PCR positive for the mobile genetic elements, class 1 integrons and SXT constins. The results of this study corroborated that deep ground water can also be an important reservoir of *V. cholerae* in plane endemic areas, suggesting a continuous monitoring of water samples for timely prevention of the disease.

Keywords Cholera · Environmental surveillance · Ground water · Integron · *Vibrio cholerae*

Introduction

Cholera is a major public health hazard for many developing countries and still continues to be an important cause of morbidity and mortality. The situation is worse in the places where proper sanitation and drinking water supply can not be assured. *Vibrio cholerae* has more than 200 serogroups, however, only O1 and O139 can cause cholera at the epidemic or pandemic scale. The other serogroups, collectively known as non O1, non O139 serogroup can cause a diarrheal disease, which is less severe than cholera and does not have epidemic potential [1]. Primarily, *V. cholerae* is an inhabitant of the aquatic environment. Hence, water plays an important role in the transmission and epidemiology of cholera. Sea water and fresh water bodies are the main reservoirs for the organism. However, cholera outbreaks occur in plane areas also where ground water is the major source of drinking water. Hence, screening of water samples for *V. cholerae*, especially drinking water sources is important to enable effective resource management and public health protection well in time. The progressive increase in antimicrobial resistance is becoming a critical area of concern and outbreak. *V. cholerae* strains are becoming multiple antibiotic resistant [2]. The rapid spread of antibiotic resistance may be attributed to the horizontal transfer of resistant genes through plasmids or integron sequences [3].

In this study, drinking ground water samples were collected from different villages of district Bhind, Central India and examined for the prevalence of *V. cholerae*. Here, we report the presence of toxigenic and pathogenic genes in these isolates and their antibiograms with the associated genetic elements.

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Materials and methods

Bacterial isolates: The water samples were collected from the hand pumps located at various places of 11 cholera affected villages of District Bhind (latitude 26°36' N, longitude 78°46' E), Madhya Pradesh, India during May–June, 2004. A total of 200 ml water sample was collected from each hand pump in sterilized container and processed as described earlier [4]. From each sample, only one representative colony was selected and stored for further studies. Reference strains of *V. cholerae* O1 and O139 were procured from National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India.

Biochemical characterization: All the bacterial isolates were screened for oxidase reaction followed by the other standard tests for presumptive identification of *V. cholerae* as described earlier [4]. Serological identification of the isolates was done by slide agglutination using commercially available polyvalent antiserum against *V. cholerae* O1 and O139 (Difco, USA). Bacterial isolates showing no agglutination with O1 or O139 antiserum were considered to belong to non O1, non O139 serogroup.

Antibiotic susceptibility: The antimicrobial susceptibility of the *V. cholerae* isolates was determined by the disc diffusion method on Mueller Hinton agar as described by the Clinical and Laboratory Standards Institutes [5]. The antibiotic discs used in this study were purchased from HiMedia, Mumbai, India.

Genomic DNA: Genomic DNA was extracted from each of the isolate using genomic DNA purification kit (MBI Fermentas, Vilnius, Lithuania). Amount and purity of DNA was determined spectrophotometrically (NanoDrop, USA). DNA extracts were stored at 4°C for further studies.

Detection of gene traits: The presence of various gene traits in *V. cholerae* was determined by a multiplex polymerase chain reaction (PCR) targeting genes for outer membrane protein (*ompW*), cholera toxin (*ctxAB*), toxin co-regulated pilus (*tcp*), zonula occludens toxin (*zot*), and somatic antigens (*rfbO1*) as described previously [6]. Briefly, PCR amplification was carried out in a 25 µl reaction mixture using a thermal cycler (BioRad, USA). The reaction mixture contained 1x reaction buffer, 200 µM dNTPs, 1.5 mM MgCl₂, 1 U of Taq polymerase, varying concentration of primers (Table 1) specific for each target gene, 100 ng of template DNA and de-ionized water. The thermal cycling conditions were: preincubation at 94°C for 2 min, 30 cycles of 1 min at 94°C for denaturation, 1 min at 59°C for annealing, 2 min at 72°C for extension, and incubation at 72°C for 10 min for final extension. In control reaction, deionized water was added to reaction mixture instead of bacterial DNA. PCR products were separated by electrophoresis us-

ing appropriate DNA ladder as a molecular size standard. All the PCR reagents used were procured from Fermentas (MBI Fermentas, Vilnius, Lithuania).

The presence of class 1 integron and SXT constins was detected by PCR using primers *qacE 1-F* and *sul1-B*, directed at the 3' CS of class 1 integrons and *int1-F* and *int1-R*, specific for SXT integrase (Table 1). Both the primers were used in the same reaction. The thermal cycling conditions were same as those in multiplex PCR.

Results and discussion

Cholera is a communicable disease due to its epidemiological behavior by which it tends to occur in explosive outbreaks. According to World Health Organization, cholera is a tragedy as theoretically, it is among the most preventable diseases but causing an estimate of more than 5 million cases each year [7]. In the present study, *V. cholerae* were isolated from the ground water in a cholera endemic area and characterized at the molecular level. A total of 8 water samples collected from different villages yielded yellow, circular, smooth and slightly flattened colonies on TCBS agar. Biochemical studies confirmed all the isolates as *V. cholerae*. However, none of the isolates gave positive agglutination tests with O1- and O139-specific antiserum, hence were considered to belong to non O1, non O139 serogroup. The major source of ground water contamination could be leakage of contaminated surface water along with hand pump pipes or water wells in a cholera endemic area. Generally, water gets stagnated around the hand pumps due to poor maintenance and unhygienic conditions. Gradually, this dirty water contaminates the ground water upon leakage. The other possible source of ground water contamination could be seepage of surface water through soil layers, when natural filtration capacity of soil fails.

All the isolates were resistant to ampicillin, ceftazidime, doxycycline, erythromycin, metronidazole, polymyxin B, roxithromycin and vancomycin. Additionally, two isolates were resistant to streptomycin. However, most of the isolates were susceptible to chloramphenicol, co-trimoxazole, gentamicin, rifampicin, nalidixic acid, streptomycin, tetracycline and trimethoprim. The antibiogram of each *V. cholerae* isolate has been given in Table 2. The results depicted the high rate of antibiotic resistance in cholera endemic areas. The pattern of antibiotic usage in a particular geographical area is an important selective force in bacterial antibiotic resistance.

Several methods have been employed for environmental detection of *V. cholerae* [8–11]. However, PCR methods have their own advantages as information about presence

of many toxigenic and pathogenic genes can be revealed simultaneously. PCR amplification of different genes yielded amplicon of variable sizes (Fig 1A, Table 1). All the isolates were found PCR positive for *ompW* gene. Out of 8 isolates, 2 were found positive for the presence of *tcp* and *zot* gene. But, no amplification products were obtained with *ctxAB* and *rfbO1* primers in any of the isolates except reference strains (Fig 1A, Table 2).

Outer membrane protein (*ompW*) gene sequence is highly conserved among *V. cholerae* strains and this is good species specific marker for different biotypes or serotypes of *V. cholerae* [12]. In this study, all isolates were positive for *ompW* gene which confirmed the biochemical results. All the isolates were PCR negative for the *rfbO1* gene.

Absence of this gene confirmed the serological tests that none of the isolates belong to O1 serogroup. In our earlier studies also, most of the environmental isolates belong to non O1, non O139 serogroup [4]. We screened the isolates for the presence of *tcp*, *ctxAB* and *zot* genes. All the isolates were negative for *ctx* gene. Previous studies have also shown that most of the environmental *V. cholerae* strains are non-cholera toxin producers [4, 13]. However, two isolates were harboring *tcp* and *zot* genes. Cholera toxin is supposed to be the most potent toxin and serves as a marker of epidemic potential among various toxins produced by *V. cholerae* [13]. But, a number of other genes are involved in cholera pathogenesis. In fact, cholera development is a multifactorial process, involving colonization of *V. cholerae*,

Fig. 1 Multiplex PCR for *ompW*, *ctxAB*, *rfbO1*, *tcp* and *zot* genes (A) and PCR for class I integron and SXT constin (B). Lane 1: *V. cholerae* O1 (Reference); Lane 2: *V. cholerae* O139 (Reference); Lane 3: BES2; Lane 4: BEV4; Lane 5: BEA1; Lane 6: BEM3; Lane 7: BEU2; Lane 8: BEP2; Lane 9: BEB4; Lane 10: BEK6, Lane M: 100 bp ladder.

Table 1 Oligonucleotide primers used in this study

Target	Nucleotide sequence	Amplicon size	Primers used per reaction	Reference
Multiplex PCR				
ompW (F)	CACCAAGAAGGTGACTTTATTGTG	304 bp	12 pmol	[6]
ompW (R)	GGTTTGTCTGAATTAGCTTCACC		12 pmol	
ctxAB (F)	GCCGGGTTGTGGGAATGCTCCAAG	536 bp	9 pmol	[6]
ctxAB (R)	GCCATACTAATTGCGGCAATCGCATG		9 pmol	
rfbO1 (F)	TCTATGTGCTGCGATTGGTG	638 bp	10 pmol	[6]
rfbO1 (R)	CCCCGAAAACCTAATGTGAG		10 pmol	
tcp (F)	CGTTGGCGGTCAGTCTTG	805 bp	12 pmol	[6]
tcp (R)	CGGGCTTTCTTCTTGTTCTG		12 pmol	
zot (F)	TCGCTTAACGATGGCGCGTTTT	947 bp	7 pmol	[6]
zot (R)	AACCCCGTTTCACTTCTACCCA		7 pmol	
Class 1 integrons				
qacEΔ1-F	ATCGCAATAGTTGGCGAAGT	800 bp	10 pmol	[19]
Sul1-B	GCAAGGCGGAAACCCGCC		10 pmol	
SXT elements				
int1-F	GCTGGATAGGTTAAGGGCGG	592 bp	10 pmol	[20]
int1-B	CTCTATGGGCACTGTCCACATTG		10 pmol	

Table 2 Presence of gene traits, mobile genetic elements and antibiogram of different *V. cholerae* isolates

Sr. No.	Place of isolation	Strain/Isolate	Year of isolation	Presence of gene traits					Mobile genetic elements		Antibiogram*
				<i>ompW</i>	<i>ctxAB</i>	<i>rfbO1</i>	<i>tcp</i>	<i>zot</i>	Class 1 integron	SXT constin	
1	Kolkata	<i>V. cholerae</i> O1	1992	+	+	+	+	+	+	-	Ery Met Pol Rox Str Van
2	Kolkata	<i>V. cholerae</i> O139	1993	+	+	-	+	+	-	+	Cot Met Pol Str Tri Van
3	Pali, Bhind	<i>V. cholerae</i> BEP2	2004	+	-	-	-	-	-	-	Amp Cef Dox Ery Met Pol Rox Van
4	Bicholi, Bhind	<i>V. cholerae</i> BEB4	2004	+	-	-	-	-	-	-	Amp Cef Dox Ery Met Pol Rox Van
5	Aethar, Bhind	<i>V. cholerae</i> BEA1	2004	+	-	-	+	+	-	-	Amp Cef Dox Ery Met Pol Rox Str Van
6	Murli Ka Pura, Bhind	<i>V. cholerae</i> BEU2	2004	+	-	-	+	+	-	-	Amp Cef Dox Ery Met Pol Rox Str Van
7	Khari Pura, Bhind	<i>V. cholerae</i> BEK6	2004	+	-	-	-	-	-	-	Amp Cef Dox Ery Met Pol Rox Van
8	Madan Ka Pura, Bhind	<i>V. cholerae</i> BEM3	2004	+	-	-	-	-	-	-	Amp Cef Dox Ery Met Pol Rox Van
9	Vijay Pura, Bhind	<i>V. cholerae</i> BEV4	2004	+	-	-	-	-	-	-	Amp Cef Dox Ery Met Pol Rox Van
10	Sher Singh Pura, Bhind	<i>V. cholerae</i> BES2	2004	+	-	-	-	-	-	-	Amp Cef Dox Ery Met Pol Rox Van

* Amp (Ampicillin, 10 µg), Cef (Ceftazidime, 10 µg), Cot (Co-trimoxazole, 25 µg), Dox (Doxycycline, 30 µg), Ery (Erythromycin, 15 µg), Met (Metronidazole, 5 µg), Pol (Polymyxin B, 50 µg), Rox (Roxithromycin, 30 µg), Str (Streptomycin, 10 µg), Tri (Trimethoprim, 5 µg), Van (Vancomycin, 30 µg)

coordinate expression of virulence factors, and toxin action. Virulence genes are also acquired in a step-by-step process [14]. Transmission of the CTX requires the presence of toxin coregulated pilus (TCP), which is essential for colonization and also encodes a transcriptional regulator that activates both *tcp* and *ctx* gene during infection [15]. Thus, TCP acts as a receptor for CTX, converting non-toxicogenic *V. cholerae* strains into toxigenic ones. This leads to the emergence of new toxigenic strains. *V. cholerae* O139 is also believed to have evolved as a result of horizontal gene transfer between the O1 and the non O1 serogroups.

In this study, two isolates were positive for *tcp* gene, indicating their potential to become toxigenic after acquiring CTX phage. Non-toxicogenic *V. cholerae* in potable water can be converted into toxigenic ones in the intestine after

consumption of water [16]. Earlier studies have also shown that environmental strains could act as reservoir for virulence genes [17]. Zonula occludens toxin is another toxin involved in cholera [13]. Two of the isolates were having this toxin gene indicating their toxigenic potential.

PCR was done for class I integron and SXT elements to find out their correlation with antibiotic resistance. Primers *qacE 1-F* and *sul1-B* yielded an amplicon of 800 bp in reference *V. cholerae* O1 strain (Fig 1B). Likewise, an amplicon of 598 bp was obtained with primers *int1-F* and *int 1-B* from reference *V. cholerae* O139 strain. However, all the environmental *V. cholerae* isolates were negative for both the mobile elements (Fig 1B, Table 2). Integrons are an important mechanism for the acquisition of antibiotic resistance genes in many bacteria. These elements are not

autonomously mobile but are able to capture, integrate and express resistance cassettes in their variable region. SXT elements confer antibiotic resistance towards chloramphenicol, sulphamethoxazole, streptomycin and trimethoprim [18]. Phenotypically also all isolates were susceptible to these antibiotics (Table 2). Thus, the study revealed that most of the environmental isolates are devoid of these genetic elements for antibiotic resistance.

In conclusion, poor sanitation practices in highly populated areas harboring endemic *V. cholerae* strains result in the contamination of ground water. Afterwards, these strains become the source of occasional outbreaks due to consumption of contaminated water and result in the emergence of new toxigenic strains for further spread of cholera. Hence, continuous environmental surveillance for the presence of *V. cholerae* in water samples is very important in cholera endemic areas to take preventive measures well in time.

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