Virulence Profile and Clonal Relationship among the *Vibrio cholerae* Isolates from Ground and Surface Water in a Cholera Endemic Area during Rainy Season

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ABSTRACT. All the *V. cholerae* non-O1, non-O139 isolates from ground and surface water samples collected during the rainy season (rainfall contributes significantly in the spread of cholera) contained *ompW* and a regulatory *toxR* gene, while many others possessed accessory cholera toxin (*ace*), hemolysin (*hlyA*) and outer membrane protein (*ompU*) genes. All the isolates lacked *ctxAB*, *tcp*, *zot*, *rfbO1* and *rfbO139* genes. The strains could be grouped into two main clusters colligating the isolates from ground water and surface water samples. The results suggest that surface water harbors various virulent *V. cholerae* strains that contaminate the ground water due to rain or poor hygienic practices, and result in the emergence of new toxigenic strains for cholera.

Abbreviations

APW	alkaline peptone water	PCR	polymerase chain reaction
BHI	brain heart infusion	TCBS	thiosulfate–citrate–bile salt–sucrose
CT	cholera toxin	*V.ch.*	*Vibrio cholerae*
NICED	*National Institute of Cholera and Enteric Diseases*		

V. cholerae has been classified in >200 serogroups. However, only two serogroups (O1 and O139) have been implicated in cholera on the epidemic or pandemic scale (Reidl and Klose 2002). The other serogroups, collectively known as non-O1, non-O139 ones are considered of negligible significance, since they have been associated with illness only in a low percentage of patients hospitalized with secretory diarrhea (Mukhopadhyay *et al.* 1996). However, toxigenic strains of *V.ch.* are rarely isolated from the environment during interepidemic periods. Hence, non-O1, non-O139 serogroup contributes to the emergence of new toxigenic and pathogenic *V.ch.* strains (Faruque *et al.* 2003; Sharma *et al.* 1998). *V.ch.* O139, a pandemic cholera strain is believed to have evolved by the horizontal gene transfer from serogroup O1 to a non-O1 serogroup (Bik *et al.* 1995).

Sea water and fresh water bodies are the main reservoirs for *V.ch.* (Lipp *et al.* 2003); it has been detected on the surfaces of aquatic organisms such as zooplankton, phytoplankton, insects, crustaceans, and plants (Colwell and Huq 1994). However, cholera outbreaks are reported from several plane areas having the ground water as major source of drinking water. Ground water contamination can be due to seepage of bacteria through the hand pumps or water wells, especially in rainy season.

Several virulence genes are reported to be present in *V.ch.* Although CT is supposed to be the major factor involved in the pathogenicity of *V.ch.*, a number of other genes are also critical for establishing a productive infection. These include regulatory gene *toxR*, outer membrane protein (*ompU*), the colonization factor toxin coregulated pilus (*tcp*), other toxin genes like zonula occludens toxin (*zot*), accessory cholera toxin (*ace*) and haemolysin (*hly*) (Kaper *et al.* 1995). More than 95 % *V.ch.* O1 and O139 strains produce CT, while the reverse is true with non-O1, non-O139 strains (Kaper *et al.* 1995). However, non-O1, non-O139 strains devoid of these toxins also cause sporadic cases of cholera (Sharma *et al.* 1998). Recently, some additional factors have also been identified in the non-O1 and/or non-O139 serogroups, which enable them to cause disease (Chen *et al.* 2007).

Here we collected ground and surface water samples from different places in rainy season from Central India and examined them for the prevalence of *V.ch.* and various associated genes; we report the virulence potential of these isolates and clonal relationship among the ground and surface water isolates.

MATERIAL AND METHODS

Bacterial cultures. The ground water (hand pumps) and surface water samples were collected from the different places on a single occasion from villages of District Morena (latitude 25°67′ N, longitude 76°67′ E), Madhya Pradesh, India in July, 2006. A 200 mL water sample was collected in a sterilized container. Before collecting the hand pump water samples, at least 2–3 L water was allowed to flow. The samples were filtered through a polycarbonate membrane of 0.2 μm pore size using vacuum pressure of 10–14 kPa. Membranes were incubated in 5 mL of APW (1 % peptone, 1 % NaCl, both *W/V*; pH 8.6) for 6 h at 37 ± 1 °C. The enriched samples were streaked onto TCBS agar plates (*Difco*, USA) and incubated overnight at 37 ± 1 °C. Subsequently, yellow, flat colonies of 1–3 mm diameter were picked up and streaked on BHI agar plates. From each sample, only one representative colony was selected and stored for further experiments. Clinical *V.ch.* were isolated from the stool samples collected from a local hospital. Reference strains of *V.ch.* O1 and O139 were procured from *NICED* (Kolkata, India).

Biochemical characterization. The isolates were screened for oxidase reaction followed by the other standard tests for presumptive identification of *V.ch.* (Tamrakar *et al.* 2006). Serological identification was done by slide agglutination using commercially available polyvalent antiserum against *V.ch.* O1 and O139 (*Difco*, USA); these reference strains were used as positive control. Isolates showing no agglutination with O1 or O139 antiserum were considered to belong to non-O1, non-O139 serogroup.

Detection of gene traits by PCR. The isolates were screened for the presence of 10 gene traits. A multiplex PCR was employed to detect genes for outer membrane protein (*ompW*), cholera toxin (*ctxAB*), toxin co-regulated pilus (*tcp*), zonula occludens toxin (*zot*), and somatic antigens (*rfbO1*) (Goel *et al.* 2007). Other genes were detected by single PCR assays. Primers were designed based on the gene sequence of *V.ch.* (*GenBank* acc. no. AB012957, AE003852.1, CP000626) using Oligo Explorer ver. 1.2 (*Gene Link*) or selected from previous papers for detection of other gene traits (Table I).

Table I. Oligonucleotide primers used

Target	Direction	Nucleotide sequence	Amplicon size, bp	Primer used[a], pmol	Reference
Multiplex PCR					
ompW	F	CAC CAA GAA GGT GAC TTT ATT GTG	304	12	Nandi *et al.* 2000
	R	GGT TTG TCG AAT TAG CTT CAC C			
ctxAB	F	GCC GGG TTG TGG GAA TGC TCC AAG	536	9	Goel *et al.* 2007
	R	GCC ATA CTA ATT GCG GCA ATC GCA TG			
rfbO1	F	TCT ATG TGC TGC GAT TGG TG	638	10	*ditto*
	R	CCC CGA AAA CCT AAT GTG AG			
tcp	F	CGT TGG CGG TCA GTC TTG	805	12	*ditto*
	R	CGG GCT TTC TTC TTG TTC G			
zot	F	TCG CTT AAC GAT GGC GCG TTT T	947	7	Singh *et al.* 2002
	R	AAC CCC GTT TCA CTT CTA CCC A			
Single PCR					
ace	F	TAA GGA TGT GCT TAT GAT GGA CAC CC	316	10	Shi *et al.* 1998
	R	CGT GAT GAA TAA AGA TAC TCA TAG G			
tox	F	CCT TCG ATC CCC TAA GCA ATA C	779	14	Rivera *et al.* 2001
	R	AGG GTT AGC AAC GAT GCG TAA G			
hlyA	F	GAG CCG GCA TTC ATC TGA AT	481	10	Singh *et al.* 2002
	R	CTC AGC GGG CTA ATA CGG TTT A			
ompU	F	CCA AAG CGG TGA CAA	655	14	*this study*
	R	TTC CAT GCG GTA AGA AGC			
rfbO139	F	TGG CAA GCG GAA GGA TTA G	554	10	*this study*
	R	CAC AAG CGG CGA CTG AAC			

[a]Per reaction.

PCR amplification was carried out in a 25 μL reaction mixture using Palm cycler (*Corbett Life Sciences*, Australia). The reaction mixture contained 1× reaction buffer, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 1 U of *Taq* polymerase (*Fermentas*, Lithuania), varying concentration of primers (Table I) specific for each target gene, template DNA and milli-Q water. The thermal cycling conditions were: pre-incubation (2 min, 94 °C), 30 cycles of denaturation (1 min, 94 °C), annealing (1 min, 57 °C), extension (2 min, 72 °C), and

final extension (10 min, 72 °C). In control reaction, deionized water was added to reaction mixture instead of bacterial DNA. PCR products were separated by electrophoresis using appropriate DNA ladder as a molecular size standard. PCR reagents used were procured from *Fermentas*.

Fingerprinting by box PCR was performed by using a single nucleotide primer BOX A1R (5′-CTA CGG CAA GGC GAC GCT GAC G-3′) (Versalovic *et al.* 1994). PCR program consisted of initial denaturation (7 min, 95 °C), 30 cycles of denaturation (1 min, 94 °C), annealing (1 min, 53 °C), extension (8 min, 65 °C), and final extension (16 min, 65 °C). The amplicons were separated by agarose gel (1.8 %) electrophoresis (60 V, 6 h). The DNA band patterns were digitized with a Gel imaging and documentation system (*Alpha Innotech*, USA) and subjected to a fingerprint analysis.

Cluster analysis of box-PCR. Digitized fingerprints were analyzed using GelCompar II (*Applied Maths*, Belgium) software, following the manufacturer's instructions. In brief, fingerprint types were defined, target lanes were searched, and gel-to-gel variations were normalized on the basis of external references (1-kb DNA stepladder; *Fermentas*) that were run at both ends for correction of distortion and normalization between gels, and within-gel common bands were aligned using internal references. The bands were selected using the auto-search function. A band-based comparison (employing the Jaccard similarity coefficient) was used to create a similarity matrix. The clustering method of Ward was used to create the dendrogram. A band position tolerance of 0.85 % was allowed in the comparison.
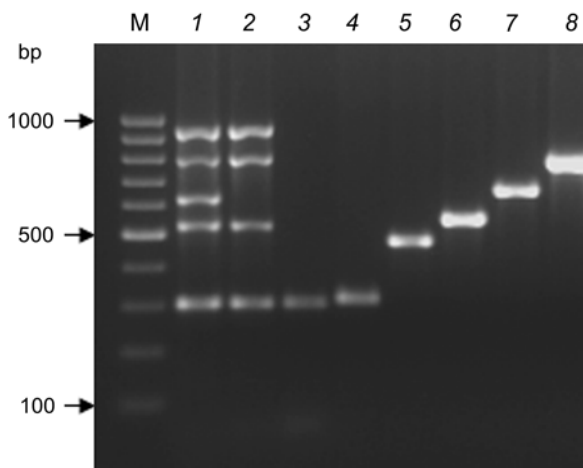
RESULTS

Bacterial isolates. A total of 18 samples (12 ground water and 6 surface water) were found positive for *V.ch.* on TCBS agar. Four isolates of *V.ch.* O1 were isolated from stool samples. Biochemical analysis confirmed all the isolates as *V.ch.* Clinical and reference strains of *V.ch.* O1 and O139 showed agglutination reactions with O1 and O139 specific antiserum. However, none of the isolates gave positive agglutination tests with these antisera, hence they were considered to belong to non-O1, non-O139 serogroup.

Gene traits. All the isolates were PCR positive for *ompW* and *toxR* gene; 3, 7 and 13 samples were PCR positive for *ace*, *ompU* and *hlyA* gene, respectively. However, none sample was positive for *rfbO1*, *rfbO139*, *ctxAB*, *tcp* and *zot* genes (Fig. 1, Table II).

Fig. 1. Agarose gel electrophoresis of multiplex PCR (lanes *1–3*) and single PCR (lanes *4–8)* products from *V. cholerae* strains;

1 – O1	*5* – ESV703 (*hlyA*)
2 – O139	*6* – O139 (*rfbO139*)
3 – ESV702	*7* – ESV703 (*ompU*)
4 – EGV727 (*ace*)	*8* – ESV703 (*toxR*)

M – 100 bp ladder (*Fermentas*).

Molecular diversity by box PCR. The box PCR of genomic DNA of isolates resulted in amplification of multiple DNA fragments of 0.55–6.0 kb length (Fig. 2). The isolates could be grouped mainly into two clusters: six isolates (ESV706, ESV707, ESV708, EGV27, EGV29, EGV50) were grouped in the same cluster, the second cluster included all the four clinical *V.ch.* O1 isolates along with the remaining surface and ground water isolates.

DISCUSSION

Among *V.ch.* strains, outer membrane protein (*ompW*) gene sequence is highly conserved; it is a good species-specific marker for different biotypes or serotypes (Nandi *et al.* 2000). All the isolates were PCR negative for the *rfbO1* and *rfbO139* gene. Serological tests also grouped all the isolates in non-O1, non-O139

V.ch. Tamrakar *et al.* (2006) have also found that most of the environmental isolates belong to non-O1, non-O139 serogroup. All the isolates were positive for *toxR* gene, a few others were positive for *ompU, ace* and *hlyA* gene (Table II).

Table II. Genotypic traits of *V. cholerae* isolates used

Isolate	Source[a]	Gene traits									
		ompW	*ctxAB*	*rfbO1*	*tcp*	*zot*	*ace*	*toxR*	*hlyA*	*ompU*	*rfbO139*
O1	–	+	+	+	+	+	+	+	+	+	–
O139	–	+	+	–	+	+	+	+	+	+	+
GCC3	CL	+	+	+	+	+	+	+	+	+	–
GCC5	CL	+	+	+	+	+	+	+	+	+	–
GCC7	CL	+	+	+	+	+	+	+	+	+	–
GCC12	CL	+	+	+	+	+	+	+	+	+	–
ESV702	SW	+	–	–	–	–	–	+	+	+	–
ESV703	SW	+	–	–	–	–	–	+	+	+	–
ESV706	SW	+	–	–	–	–	–	+	–	–	–
ESV707	SW	+	–	–	–	–	–	+	–	+	–
ESV708	SW	+	–	–	–	–	–	+	–	+	–
ESV709	SW	+	–	–	–	–	–	+	+	–	–
EGV721	GW	+	–	–	–	–	–	+	+	–	–
EGV727	GW	+	–	–	–	–	+	+	+	–	–
EGV729	GW	+	–	–	–	–	–	+	–	–	–
EGV731	GW	+	–	–	–	–	+	+	+	–	–
EGV732	GW	+	–	–	–	–	–	+	+	–	–
EGV733	GW	+	–	–	–	–	–	+	+	–	–
EGV737	GW	+	–	–	–	–	–	+	+	+	–
EGV738	GW	+	–	–	–	–	+	+	+	–	–
EGV750	GW	+	–	–	–	–	–	+	–	–	–
EGV752	GW	+	–	–	–	–	–	+	+	+	–
EGV753	GW	+	–	–	–	–	–	+	+	+	–
EGV754	GW	+	–	–	–	–	–	+	+	–	–

[a]CL – clinical, SW – surface water, GW – ground water.

Gene *toxR* is a regulator of virulence in *V.ch.*, activating *toxT* promoter; ToxT acts downstream to regulate various promoters of toxigenic and pathogenic genes (Dirita *et al.* 1991). ToxR also regulates the expression of *ompU* gene (Crawford *et al.* 1998). OmpU protects *V.ch.* against bile sensitivity, addition of bile can increase the level of OmpU in the outer membrane (Provenzano *et al.* 2000). Thus, OmpU is involved in conferring bile resistance, which may be required for survival in the human gastrointestinal tract. Thus, several of our strains had the potential to survive in the human gut. All the *V.ch.* non-O1, non-O139 strains used by us lacked the known virulence traits associated with toxigenic *V.ch.* O1, O139. All the isolates were negative for *ctxAB* and *tcp* genes, both of which are recognized as important components contributing to the pathogenicity of toxigenic strains O1 and O139. However, all the non-O1, non-O139 strains examined here possessed the gene encoding the regulatory protein ToxR, which controls the coordinate expression of genes associated with pathogenicity in toxigenic *V.ch.* O1 and/or O139.

Thirteen strains were PCR positive for hemolysin (*hlyA*) gene, a virulence factor associated with *V.ch.* strains (Yamamoto *et al.* 1984). Traditionally, hemolysin is used to differentiate between classical and El Tor strains of *V.ch.* Sharma *et al.* (1998) and Yamamoto *et al.* (1986) have also shown that most of the non-O1 and/or non-O139 isolates are *hlyA* positive. Hemolysin produced by *V.ch.* non-O1 is indistinguishable biologically, physico-chemically and antigenically from the hemolysin produced by El Tor strains (Yamamoto *et al.* 1986). (The hemolysin was found in bloody fluid accumulation in ligated rabbit ileal loops; Kaper *et al.* 1995.)

Three isolates contained accessory cholera toxin (*ace*) gene, which is also involved in significant fluid accumulation in rabbit ligated ileal loop (Kaper *et al.* 1995). All the isolates lacked *ctxAB, tcp* and *zot* genes, associated with pathogenicity of *V.ch.* Some papers (Bidinost *et al.* 2004; Kaper *et al.* 1995; Tamrakar *et al.* 2006) have also shown that most of the environmental *V.ch.* strains are non-cholera toxin producers.

Box PCR was used to measure the relatedness among strains isolated from both surface and ground water sources and relatedness with clinical *V.ch.* O1 isolates, all showing two main genetic profiles coexisting in the environment. Clinical *V.ch.* O1 strains produced the same fingerprinting pattern and were grouped

in the same cluster along with several other surface and ground water isolates (Fig. 2). Although, the strains were diverse, no significant difference has been observed between the surface-water and ground-water isolates. The isolates from both sources were closely related, indicating the contamination of ground water from surface water. This further shows that most of non-toxigenic clones are divers and transduction process in the natural environment converts these strains into toxigenic ones.
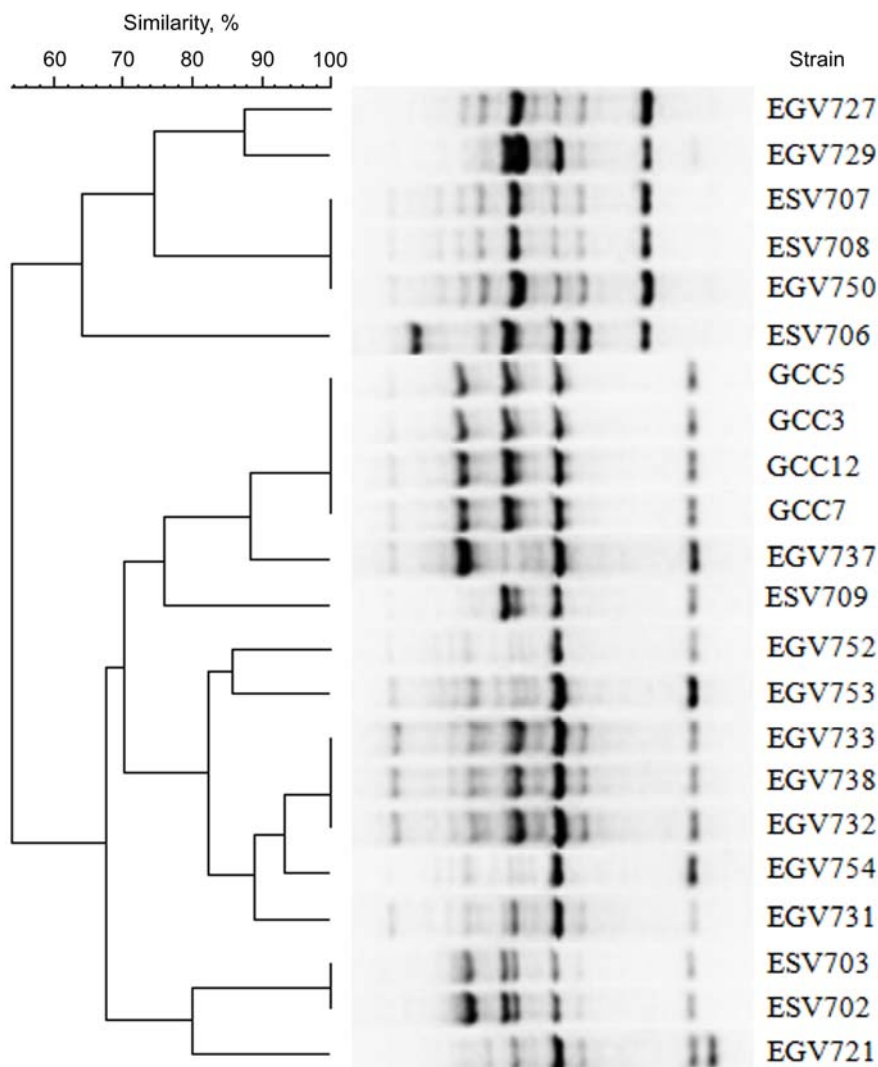

Fig. 2. Dendrogram of *V. cholerae* non-O1, non-O139 strains based on box PCR fingerprinting pattern.

There is a close relationship between the *V.ch.* isolates from surface and ground water. Surface water harbors strains possessing various types of virulence genes, which subsequently reach the drinking ground water by rain or poor sanitation practices. 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.ch.* in water samples is very important in cholera endemic areas to take preventive measures well in time.

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