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Detection of Virulence Genes in *Vibrio cholerae* Isolated from Aquatic Environment in Kerala, Southern India

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Abstract *Vibrio cholerae* is the etiologic agent of cholera. It is an autochthonous inhabitant of all aquatic environments. The virulence of *V. cholerae* is maintained by the CTX genetic element and *tcpA* gene. In the present investigation, environmental strains of *V. cholerae* isolated from different aquatic biotopes in Kerala were identified and serotyped. The antibiotic resistance pattern and presence of virulence and regulatory genes were examined. We found the presence of toxigenic non-O1/non-O139 strains harboring the CTX genetic element, heat-stable enterotoxin, *rtxA* gene, El Tor hemolysin, and *Vibrio* pathogenicity island (VPI). The strains also produced the cholera toxin (CT) as determined by monosialoganglioside enzyme-linked immunosorbent assay. A few strains belonging to the O1 serogroup but lacking the CTX genetic element were also observed. The majority of the environmental strains belonged to non-O1/non-O139 serogroup with many possessing *toxR*, *ompU*, heat-stable enterotoxin, and *rtxA* gene. The toxigenic non-O1/non-O139 strains exhibited resistance to trimethoprim, ampicillin, and polymixin B and intermediate resistance to co-trimoxazole. However, all other environmental strains were found resistant to ampicillin and polymixin B. Our findings demonstrate that the virulence genes are dispersed among the environmental strains of *V. cholerae* and a complex aquatic environment can give rise to pathogenic *V. cholerae.*

Keywords *Vibrio cholerae* · Cholera toxin · Toxin-coregulated pilus · *RTX* gene · Heat-stable enterotoxin

Introduction

Cholera is characterized by a severe watery diarrhea caused by toxigenic *Vibrio cholerae*, which colonizes the small intestine of humans and produces an enterotoxin, cholera toxin

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(CT). The outbreak of cholera is very frequent in Kerala, which is known for its extensive backwater system [1]. The genes encoding CT together with other toxins viz. zonula occludens toxin (*Zot*), accessory cholera enterotoxin (*Ace*), and core-encoded pilin (*Cep*) are carried on the genome of a filamentous bacteriophage (CTXΦ). The CTXΦ uses the toxin-coregulated pilus (TCP) as a receptor, and hence, the expression of TCP by the bacterium is a prerequisite for its susceptibility to the phage. Thus, a virulence factor of the bacterium in human serves as a receptor for CTXΦ. In the present study, the main objectives are to explore the presence of virulence and regulatory genes and the nature of antibiotic resistance in environmental strains of *V. cholerae* isolated from the epidemic-prone aquatic environment of Kerala. The expression of CT by the toxigenic environmental strains was also detected by GM_1 ELISA.

Materials and Methods

Sample Collection and Processing

Multiple water samples were collected from Trivandrum (well water and sewage water), Alappuzha (estuarine water), and Kottayam (riverine water) districts in Kerala, Southern India where outbreaks of cholera have been reported previously [1]. For sample collection, 1 l of water was filtered successively through Whatman membrane no. 1 and 0.22 μm membrane (Millipore). The retained contents on the membrane filter were washed into phosphate-buffered saline (pH 7.4). Samples were enriched in alkaline peptone water (APW) at 37 °C for 6 to 8 h. Approximately 5 μl of enriched APW broth was streaked by using an inoculating loop onto thiosulfate–citrate–bile salts–sucrose agar (Difco) and incubated at 37 °C for 18–24 h. The suspected colonies were picked up and subjected to biochemical test and polymerase chain reaction (PCR).

Bacterial Strains

A total of 50 environmental strains were included in the present study. Forty-eight strains were non-O1/non-O139 (46 *ctx* −ve and 2 *ctx* +ve strain) and 2 O1 strain (*ctx* −ve). *V. cholerae* El Tor strain 20, biotype classical strain 569B, *V. cholerae* O139 strain ATCC 51394, and NAG 688 were used as the PCR positive controls.

PCR Analysis

The bacterial cell lysate was used as a template for PCR analysis. The *V. cholerae* strains grown overnight at 37 °C in Luria Bertani broth (USB, Amersham Life Technology) were boiled, snap-cooled, and stored at −20 °C until use. The various oligonucleotide sequences used in this study are shown in Table 1. The amplification of *V. cholerae* species-specific conserved intergenic spacer region between 16S and 23S rRNA genes as carried out by the method of Chun et al. [2]. The *rfb* genes specific for *V. cholerae* O1, O139 serogroups, and the *ctxA*-encoding subunit A of CT were amplified by using multiplex PCR as carried out by the method of Hoshino et al. [3]. The hexaplex PCR for rapid detection of virulence and regulatory genes viz. *ctxA*, *zot*, *ace*, *tcpA*, *ompU*, and *toxR* as carried out by the method of Singh et al. [4]. The presence of *ctxB* and *hlyA* in *V. cholerae* strains were determined by a PCR assay followed by the method of Olsvik et al. and Rivera et al. [5, 6]. The detection of *rtxA* and *st* genes were carried out using specific PCR for which amplification of the target

Table 1 Sequences of primers used for PCR analysis.

Sl. no.	Primers	Nucleotide sequence (5′–3′)	Amplicon size (bp)	Reference
1	*ISR*—F	TTAAGCSTTTTCRCTGAGAATG	295–310	[2]
	ISR—R	AGTCACTTAACCATACAACCCG		
2	*O1rfb*—F	GTTTCACTGAACAGATGGG	192	[3]
	O1 rfb—R	CGGTCATCTGTAAGTACAAC		
3	*O139 rfb*—F	AGCCTCTTTATTACGGGTGG	449	[3]
	O139 rfb—R	GTCAAACCCGATCGTAAAGG		
4	*ctxA*—F	CGGGCAGATTCTAGACCTCCTG	564	[10]
	ctxA—R	CGATGATCTTGGAGCATTCCCAC		
5	*ctxB*—F	GATACACATAATAGAATTAAGGATG	460	[4]
	ctxB—R	GGTTGCTTCTCATCATCGAACCAC		
6	*zot*—F	TCGCTTAACGATGGCGCGTTTT	947	[4]
	zot—R	AACCCCGTTTCACTTCTACCCA		
7	*ace*—F	TAAGGATGTGCTTATGATGGACACCC	316	[4]
	ace—R	CGTGATGAATAAAGATACTCATAGG		
8	*ompU*—F	ACGCTGACGGAATCAACCAAAG	869	[4]
	ompU—R	GCGGAAGTTTGGCTTGAAGTAG		
9	*tcpA*—F	CACGATAAGAAAACCGGTCAAGAG	620	[4]
	tcpA—R/Clas	TTACCAAATGCAACGCCGAATG		
	tcpA—R/El Tor	CGAAAGCACCTTCTTTCACACGTTG		
10	*toxR*—F	CCTTCGATCCCCTAAGCAATAC	779	[4]
	toxR—R	AGGGTTAGCAACGATGCGTAA		
11	*hlyA*—F/El Tor	GGCAAACAGCGAAACAAATACC	481	[6]
	hlyA—F/Clas	GAGCCGGCATTCATCTGAAT	738/727	
	hlyA—R	CTCAGCGGGCTAATACGGTTTA		
12	*rtxA*—F	GGGATACAATGCCCTCTGGCA	977	Present study
	rtxA—R	TGGGTTGGCGGTTGGATTTTAC		
13	*st*—F	TATTATTTTCTTCAATCGCATTTAGC	206	Present study
	st—R	ATTTAAACATCCAAAGCAAGCTGG		

DNA was carried out in a thermal cycler (Master cycler gradient, Eppendorf) using 200 μl PCR tubes with a reaction mixture volume of 25 μl.

Detection of CT by GM_1 ELISA

To detect the expression of CT by environmental strains, the cells were grown in AKI medium (containing bactopeptone, 15 g; NaCl, 5 g; yeast extract, 5 g; sodium bicarbonate, 3 g; per liter; pH 7.5) at 37 °C with shaking for 16 h. After centrifugation, the supernatant was examined for the presence of CT by a monosialoganglioside enzyme-linked immunosorbent assay (GM_1 ELISA) as carried out by the method of Svennerholm and Holmgren [7]. The pure CT obtained from List Biological Laboratories, UK was used as the positive control.

Antibiotic Susceptibility Tests

All the *V. cholerae* O1 strains were tested for antimicrobial resistance with antibiotic impregnated discs (Hi-Media Laboratories, Bombay, India). The following antibiotic discs with concentration of the drug as stated in the parentheses were used: ampicillin (A, 10 μg),

chloramphenicol (C, 30 μg), cefotaxime (Ce, 30 μg), ciprofloxacin (Cf, 5 μg), cephalexin (Cp, 30 μg), co-trimoxazole (Co, 25 μg), furazolidone (F, 100 μg), gentamycin (G, 10 μg), neomycin (N, 30 μg), norfloxacin (Nx, 10 μg), nalidixic acid (Na, 30 μg), polymixin B (Pb, 50 U), streptomycin (S, 10 μg), spectinomycin (Se, 100 μg), tetracycline (T, 30 μg), trimethoprim (Tr, 5 μg). The control strain was *Escherichia coli* ATCC 25922. *V. cholerae* strains were characterized as susceptible, intermediate, or resistant based on the diameter of the inhibition zones around the disc according to the manufacturer's instructions.

Results and Discussion

The virulence profiles of the various environmental isolates of *V. cholerae* are shown in Table 2. Out of 50 strains analyzed, 48 strains belonged to the non-O1/non-O139 serogroup with 2 strains possessing the CTX genetic element (Fig. 1). This highlights the importance of non-O1/non-O139 *V. cholerae* with respect to the presence of virulence genes. The non-O1/non-O139 environmental strains carrying the CTX genetic element are extremely rare and such strains causing fatal outbreak is well-documented [8]. Two strains belonging to the O1 category but lacking the *ctx* gene cluster were also reported in the present investigation.

The result of hexaplex PCR revealed that majority of environmental isolates possesses *toxR* genes and *ompU* genes (90% *toxR*+^{ve} and 80% ompU+^{ve}) (Fig. 2). The toxigenic non-O1/non-O139 strains were found to be positive for *ctxA*, *zot*, *ace*, and *toxR* genes and negative for *tcpA*. The failure to amplify *tcpA* may be because of polymorphism in toxigenic strains (A199 and A217) which could not be detected by primers specific to El Tor and the classical type of *tcpA*. Several variants of *tcpA* have been reported previously with divergence of sequence in the carboxy-terminal half [9], whereas other genes of VPI viz. *tcpC*, *tcpD*, *tcpE*, *tcpT*, *tcpI*, and *acfB* were found to be present (data not shown). The absence of the *ompU* gene was also noted in toxigenic strains. The O1 strains (A181and A182) isolated belonged to the El Tor biotype (as the *tcpA* El Tor type gene was present). The other genes like *zot*, *ace*, and *toxR* and genes of VPI were found to be present (Table 2). Such strains are possible progenitors or intermediates in the origination of toxigenic strains as they possess the *tcpA* gene and are capable of acquiring CTXΦ by horizontal gene transfer. It has been shown that *tcp* +ve O1 strains are more likely to be infected with CTXΦ [10]. The toxigenic non-O1/non-O139 strains were also positive for *ctxB*.

Of the strains, 64% and 16% showed positive results for the presence of the *rtxA* gene and heat-stable enterotoxin, respectively. The *rtxA* has been previously correlated with the cytotoxic activity that causes mammalian Hep-2 cells to detach and round up [11]. The heat-stable enterotoxin has been reported to cause diarrhea during an outbreak in Thailand [12]. Eighty-four percent of the environmental strain was found to harbour El Tor like *hlyA*, whereas the remaining possessed the classical type. The previous studies have indicated that *V. cholerae* hemolysin exhibits vacuolating activity on nucleated mammalian cells [13]. The combination of all these toxins or alone may be responsible for the occasional diarrhea caused by nontoxigenic (NT) non-O1/non-O139 strains.

In the present study, the toxigenic strains from the environment produced CT in the range of 200–1,000 pg/ml when they were cultured in AKI medium. This indicates that the ability to secrete CT is not lost even if the strains are isolated from aquatic environment and the strains are virulent possessing epidemic potential. The antibiogram profiles revealed that the toxigenic non-O1/non-O139 strains exhibited resistance to ampicillin, trimethoprim, and polymixin B and intermediate resistance to co-trimoxazole. The presence of such

Table 2 Virulence profile of environmental isolates of *V. cholerae*.

Sl. no.	Strain	*ctxA*	*ctxB*	*ace*	*zot*	*rtxA*	*st*	*hlyA*(El Tor)	*tcpA*	*toxR*	*ompU*
1	A199	+	+	+	+	+	+	+	−	+	−
2	A217	+	+	+	+	+	+	+	−	+	−
3	A181	−	−	+	+	+	+	+	+	+	+
4	A182	−	−	+	+	+	+	+	+	+	+
5	A196	−	−	−	−	+	−	−	−	−	+
6	A198	−	−	−	−	−	−	+	−	+	−
7	A200	−	−	−	−	−	−	+	−	+	+
8	A201	−	−	−	−	−	−	+	−	+	−
9	A202	−	−	−	−	+	+	+	−	+	+
10	A205	−	−	−	−	+	−	−	−	+	+
11	A206	−	−	−	−	+	−	+	−	+	+
12	A207	−	−	−	−	−	−	+	−	+	+
13	A208	−	−	−	−	+	−	+	−	+	−
14	A209	−	−	−	−	+	−	+	−	+	+
15	A212	−	−	−	−	−	−	+	−	−	+
16	A216	−	−	−	−	+	−	+	−	+	+
17	A217	−	−	−	−	−	−	+	−	+	+
18	K45	−	−	−	−	−	−	−	−	+	+
19	K46	−	−	−	−	+	−	+	−	+	+
20	K50	−	−	−	−	+	+	+	−	+	+
21	K51	−	−	−	−	−	−	+	−	+	+
22	K53	−	−	−	−	+	−	+	−	+	+
23	K56	−	−	−	−	+	−	+	−	+	+
24	K57	−	−	−	−	+	−	−	−	+	+
25	K59	−	−	−	−	−	−	+	−	+	+
26	K61	−	−	−	−	+	−	+	−	+	+
27	K66	−	−	−	−	+	−	+	−	+	+
28	K67	−	−	−	−	+	−	+	−	+	+
29	K68	−	−	−	−	−	−	+	−	+	+
30	K69	−	−	−	−	+	−	+	−	+	+
31	K70	−	−	−	−	+	−	+	−	+	+
32	K72	−	−	−	−	+	−	+	−	+	+
33	K73	−	−	−	−	+	+	+	−	+	−
34	K75	−	−	−	−	+	−	+	−	+	+
35	T61	−	−	−	−	−	−	−	−	−	+
36	T66	−	−	−	−	+	−	+	−	+	−
37	T104	−	−	−	−	−	−	−	−	+	+
38	T120	−	−	−	−	+	−	+	−	+	+
39	T124	−	−	−	−	−	−	+	−	−	−
40	T125	−	−	−	−	+	−	−	−	−	+
41	T127	−	−	−	−	−	−	+	−	+	+
42	T130	−	−	−	−	−	−	+	−	+	+
43	T132	−	−	−	−	+	−	−	−	+	−
44	T134	−	−	−	−	+	−	+	−	+	+

1–17: strains collected from Alappuzha, *18–34*: strains collected from Kottayam, *35–50*: strains collected from Trivandrum, +: positive, −: negative

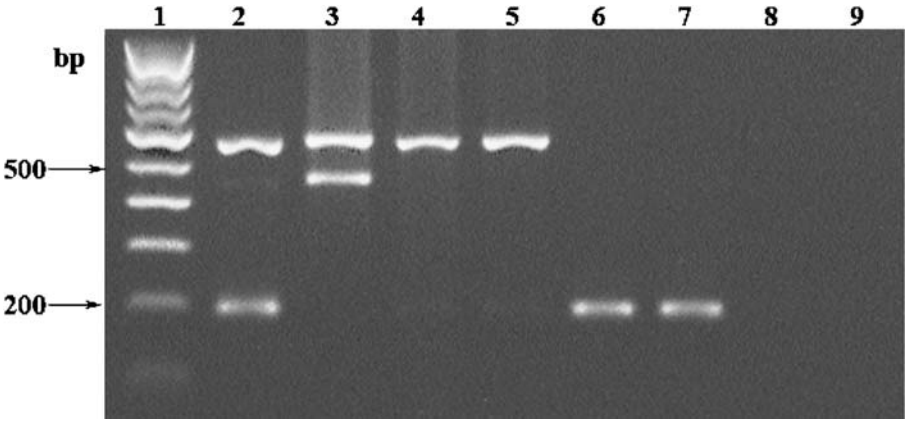

Fig. 1 Agarose gel electrophoresis of multiplex PCR products from *V. cholerae* environmental strains. *Lane 1* 100 bp ladder, *lane 2* VC20 O1 El Tor, *lane 3 V. cholerae* O139 strain ATCC 51394, *lane 4* A199, *lane 5* A217, *lane 6* A181, *lane 7* A182, *lanes 8–9*, non-O1/non-O139 strains

multidrug-resistant strains in the environment is a potential threat in the context of public health. It was interesting to note that resistance to trimethoprim was not linked to SXT conjugative element as confirmed by PCR (data not shown) whereas other strains were found to be resistant to ampicillin and polymixin B.

In this paper, we report the presence of toxigenic strains with epidemic potential belonging to the non-O1/non-O139 serogroup in the epidemic-prone aquatic environment of Kerala. Such toxigenic strains seem to survive during the interepidemic period as no epidemics were reported during the period of the present investigation. Thus, the non-O1/non-O139 strains can no longer be ignored, as some strains possess the ability to precipitate cholera-like syndrome and finally flare up into localized outbreak. There has been an escalation in interest in the non-O1/non-O139 serogroup following the discovery of the

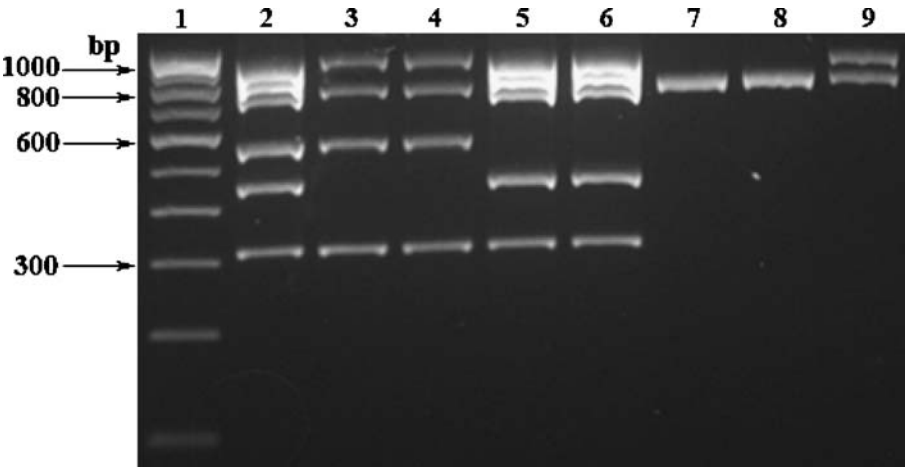

Fig. 2 Agarose gel electrophoresis of hexaplex PCR products from *V. cholerae* environmental strains. *Lane 1* 100 bp ladder, *lane 2* VC20 O1 El Tor strain, *lane 3* A199, *lane 4* A217, *lane 5* A181, *lane 6* A182, *lanes 7–9*, non-O1/non-O139 strains

O139 serogroup [14] and following the finding that environmental strains play an important role in the evolution of toxigenic *V. cholerae*. Several localized outbreaks of diarrhea caused by NT non-O1/non-O139 serogroup have been described [12, 15, 16]. The presence of NT O1 strains in the environment is extremely significant since they may emerge as a new clone of toxigenic *V. cholerae* by acquisition of CTXΦ. The continuous emergence of new toxigenic clones in the environment may lead to epidemic outbreaks of cholera and, hence, continuous monitoring of this pathogenic bacterium is highly warranted in the context of public health.

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