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Molecular characterization of *Vibrio cholerae* non-O1/non-O139 isolated from coastal water of Arabian Gulf

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The mechanism of pathogenic *Vibrio cholerae* to cause disease depends on the expression of virulence factors such as a potent cholera toxin (CT), and pilus colonization factor (toxin co-regulated pilus; TCP). The gene's molecular characterization provides *V. cholerae* ecology of the genes information about the ecology of *V. cholerae* which is an autochthonous inhabitant of aquatic environment as well as pathogenic for humans. The *ctxAB* and *tcpA* genes are known to play a significant role in maintaining virulence in *V. cholerae*, and these genes are believed to be exclusively associated with clinical strains of O1 and O139 serogroups. In this study, we examined the presence of virulence genes, including *ctxA* and *tcpA* (Classical and El Tor), as well as *toxR* and the genes responsible for O-antigen biosynthesis and for generation of serotype-specific determinants located in the *rfb* region in the *V. cholerae* chromosome. Out of 23 suspected *V. cholerae* isolated from Arabian Gulf coastal water of the eastern province of Saudi Arabia only one isolate was detected and harbored *tcpA* (El Tor), O1*rfb* and *toxR* genes. This study demonstrates the transfer of virulence genes among environmental strains of *V. cholerae* which appears to constitute an environmental reservoir of virulence genes, thereby providing new insights into the *V. cholerae* ecology. To the best of our knowledge, this is the first report of the isolation of *V. cholerae* from Arabian Gulf coastal water environment.

Key words: *Vibrio cholerae* non-O1/non-O139, virulence genes, PCR, Arabian Gulf water.

INTRODUCTION

The worldwide re-emergence of *Vibrio cholerae* has posed a challenge for microbiology and epidemiological surveillance of cholera. Over the last few years, many studies that increased the understanding of *V. cholerae* ecology pathogenesis and epidemiological behaviour had been conducted (Faruque et al., 1998). *V. cholera* is a

natural inhabitant of freshwater, estuarine and seawater as a reservoir and source of its transmission (Colwell, 2004). Among the many causal organisms, only *V. cholerae* has caused repeated pandemics dating from the pandemics of the 19th century to recent major epidemics in South America and Africa. *V. cholerae* is subdivided into more than 208 somatic O-antigen serogroups, but only two serogroups, O1 and O139, are recognized as pathogenic for humans and as responsible for epidemic and pandemic cholera. In contrast, non-O1/non-O139 serogroups of *V. cholerae* had been associated with

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sporadic cases of gastroenteritis, septicemia and extra-intestinal infections (Dhar et al., 2004). Only two serogroups O1 and O139 had been associated with epidemic cholera. However, numerous outbreaks of diarrhoea caused by non-O1, non-O139 serogroups of *V. cholerae* had been reported (Sharma et al., 1998).

The pathogenic variants of *V. cholerae* express genes encoding virulence-associated factors; in order to cause diarrhoea, strains require genes for the cholera toxin, the colonization factor TCP (toxin-coregulated pilus) and the central regulatory protein ToxR. Regarding the pathogenicity mechanisms of *V. cholerae* non-O1 and non-O139, different virulence factors had been suggested to be involved in the disease caused by this pathogen (Morris, 1994). Although some of these strains produce the cholera toxin (CT) or a cholera-like toxin, most of them lack the CTX ϕ phage and the toxin coregulated factor (TCP) and even the complete Vibrio Pathogenicity Island (VPI). Even if different studies on *V. cholerae* O1 and O139 indicate that CT, TCP and ToxR (the regulator of expression and secretion of CT and TCP) are necessary to cause diarrhoea, there are studies in which nontoxigenic environmental isolates of *V. cholerae* show a secretory response in intestinal tissue that may be caused by other virulence factors (Datta-Roy et al., 1986). On the other hand, *V. cholerae* non-O1, non-O139 produces other extracellular products such as the heat-stable toxin, the hemolysin and the pore-forming toxin that may be responsible for the pathogenesis of these strains.

It has become evident from earlier reports that a new epidemic *V. cholerae* clone could emerge by acquisition and combination of crucial virulence genes from the environmental pool through horizontal gene transfer by phages and other mobile elements had been demonstrated. In fact, non-O1 and non-O139 strains may act as reservoirs of virulence genes in the environment which could lead to the emergence of new epidemic or virulent variants by 'mixing and matching' genes in the environment or in the human intestine (Chakraborty et al., 2000). Cholera surveillance is now under way in many countries, based primarily on the detection of *V. cholerae* O1 and O139 and determines the presence of cholera toxin using biological and molecular methods. However, virulence-associated factors in *V. cholerae* isolates from environmental sources are of concern.

The main objective of this study was to investigate the presence of virulence genes as potential pathogenic markers suitable for monitoring environmental vibrios strains isolated from the Arabian Gulf coastal water of eastern province of Saudi Arabia.

MATERIALS AND METHODS

Bacterial strains

From our previous study on the prevalence of clinically significant of pathogenic *Vibrio* spp on the Arabian Gulf coastal water of the

eastern province of Saudi Arabia between February 2009 and January 2010 (unpublished data), a total of 23 isolates of *V. cholerae* non-O1/non-O139 were isolated from seawater samples. All the isolates of *V. cholerae* were confirmed by using biochemical tests, API 20 E identification system, and antisera for O1 polyvalent and O139.

DNA extraction

A modification of the Murray and Thompson method (Murray and Thompson, 1980) was used for DNA extraction. In brief, cells from an 18 h LB culture were collected and re-suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), treated with 10% (wt/vol) sodium dodecyl sulfate and freshly prepared proteinase K (Sigma Chemical Co., St. Louis, Mo.), and incubated at 37°C for 1 h. After incubation, 10% cetyl trimethyl ammonium bromide in 0.7 M NaCl was added and incubated at 65°C for 10 min. The aqueous phase was treated with phenol-chloroform, and the DNA pellet was washed with 70% ethanol. The extract nucleic acid was suspended in TE and treated with RNase at 37°C for 30 min.

PCR protocol

Six pairs of primers (*ctxA*, *tcpA* [classical], *tcpA* [EI Tor], *O1rfb*, *O139rfb*, and *toxR*) were used in this study are shown in Table 1 (Keasler et al., 1993). On each of the samples, two sets of multiplex PCRs were performed to determine the presence of five established virulence genes of *V. cholerae* O1 and O139. The first set was performed to determine the presence of *tcpA* (Keasler et al., 1993) (gene encoding the major structural subunit of toxin coregulated pilus) and *ctxA* (Shirai et al., 1991) (gene encoding the enzymatic subunit of CT). The second set was performed to determine the presence of genes of the *rfb* (genes for surface antigen) regions of O1/O139 and *ctxA* (Hosino et al., 1998). PCR was also performed to determine the presence of *V. cholerae* *toxR* (regulatory gene) (Miller et al., 1998). The PCR reaction was performed in a buffer containing 10 μ l of 10 \times PCR amplification buffer [500 mM KCl, 100 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 150 mM MgCl₂], 8 μ l of dNTPs (2.5 mM each); 100 pmol of each primers and 4 U of *Taq* DNA polymerase (Promega, USA). PCR was carried out in 0.2 ml PCR tubes with 23 μ l of the mixture described above and 2 μ l of the template DNA. PCR assays were performed using an automated thermal cycler (Applied Biosystems, USA). The PCR conditions consisted of a preincubation step for 10 min at 94°C and a termination step with a final extension for 10 min at 72°C, with a middle step consisting of 30 cycles of 1.5 min at 94°C, 1.5 min at 60°C, 1.5 min at 72°C (for *tcpA* of classical biotype and *tcpA* of EI Tor biotype); 35 cycles of 1.5 min at 94°C, 1.5 min at 60°C and 1.5 min at 72°C (for *ctxA*); 35 cycles of 1.5 min at 94°C, 1.5 min at 60°C and 1.5 min at 72°C (for O1 *rfb* and O139 *rfb*); 25 cycles of 40 s at 94°C, 40 s at 64°C and 1 min at 72°C (for *toxR*).

RESULTS AND DISCUSSION

A total of 23 environmental isolates of the genus *V. cholerae* isolated from Arabian Gulf water of the eastern province of Saudi Arabia were screened for the presence of the five virulence genes (*ctxA*, *tcpA* (Classical), *tcpA* (EI Tor), *O1rfb*, *O139rfb* and *toxR*) in the present study. The results showed that the cholera toxin gene was absent in all the isolates (*ctxA*). Out of the 23 isolates only one isolate of *Vibrio cholerae* non-O1/non-O139

Table 1. Primers used in this study.

Target gene	Primer pair	Primer sequence (5'- 3')	Amplicon size (bp)	Reference
ctxA	<i>ctxA</i> -F <i>CtxA</i> -R	5'-CTCAGACGGGATTTGTTAGGCACG-3' 5'-TCTATCTCTGTAGCCCCTATTACG-3'	301	Shirai et al. (1991)
<i>tcpA</i> (Classical)	<i>tcpA</i> (Classical)-F <i>TcpA</i> (Classical)-R	5'-CACGATAAGAAAACCGGTCAAGAG-3' 5'-ACCAAATGCAACGCCGAATGGAGC-3'	617	Keasler et al. (1993)
<i>tcpA</i> (El Tor)	<i>tcpA</i> (El Tor)-F <i>tcpA</i> (El Tor)-R	5'-GAAGAAGTTTGTAAGAAGAAGAACAC-3' 5'-GAAAGGACCTTCTTTCACGTTG-3'	471	Keasler et al. (1993)
O1<i>rfb</i>	O1 <i>rfb</i> -F O1 <i>rfb</i> -R	5'-GTTTCACTGAACAGATGGG-3' 5'-GGTCATCTGTAAGTACAAC-3'	449	Hosino et al. (1998)
O139<i>rfb</i>	O139 <i>rfb</i> -F O139 <i>rfb</i> -R	5'-AGCCTCTTTATTACGGGTGG-3' 5'-GTCAAACCCGATCGTAAAGG-3'	192	Hosino et al. (1998)
<i>toxR</i>	<i>ToxR</i> -F <i>ToxR</i> -R	5'-CGGGATCCATGTTCCGGATTAGGACAC-3' 5'-CGGGATCCTACTCACACACTTTGATGGC-3'	900	Miller et al. (1987)

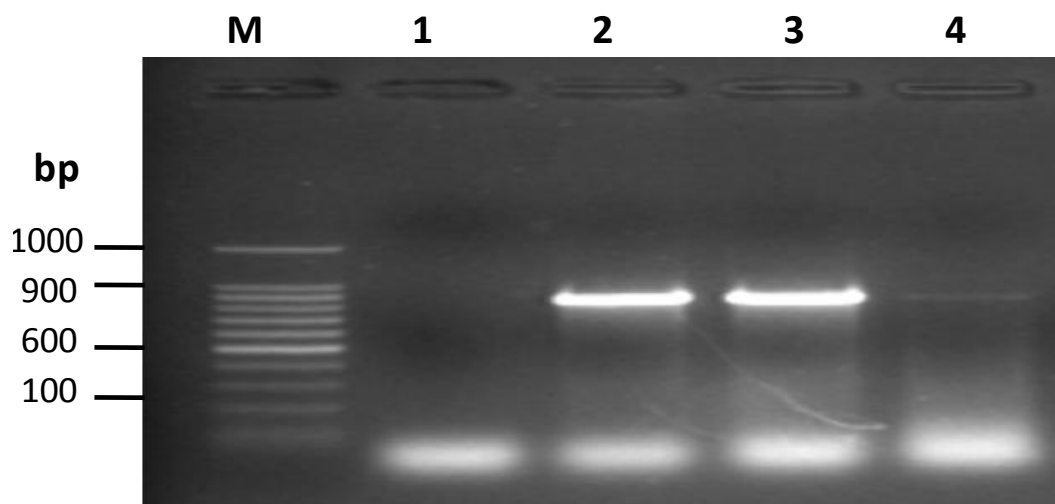


Figure 1. PCR analysis for *toxR* (900 bp) of strains used in this study. Lanes: M, 100 bp ladder; 1, negative control; 2, *V. cholerae* strain (positive control); 3, *V. cholerae* non O1/non O139 strain VC-12; 4, *V. cholerae* non-O1/O139 strain VC-16.

(isolated strain VC-12) was found to be positive for *tcpA* (El Tor), O1*rfb* and *toxR* genes as shown in Figure 1 and 2.

Cholera toxin (CT) is encoded by a transferable filamentous phage (CTX Φ) and reports had implied the acquisition of these CT genes under analogous conditions to those in the aquatic environments (Faruque and Nair, 2002). *ctxAB* operon, which encodes for the A and B subunits of CT, resides in the genomes of CTX Φ .

All *Vibrio* strains proficient of causing cholera perpetually carry genes for TCP which is an adhesin that is coordinately regulated with CT production (Taylor et al., 1987). TCP is the solitary *V. cholerae* pilus that has been demonstrated to have a role in colonization of the human's gut mucous and of infant mice (Taylor et al., 1987), the latter being an experimental model. It has been presumed that CT and TCP are exclusively associated with clinical strains of *V. cholerae*, notably

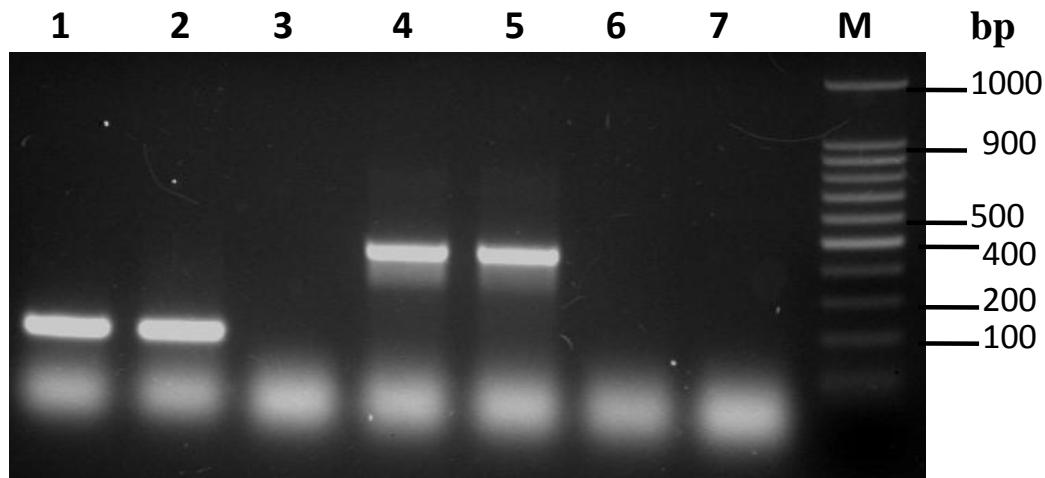


Figure 2. PCR analysis of *O1rfb* (192 bp), *tcpA* of El Tor type (471 bp) used in this study. Lanes: 1, *V. cholerae* non-O1/non-O139 strain VC-12; 2, positive control; 3, negative control; 4, Positive control; 5, *V. cholerae* non-O1/non-O139 strain VC-12; 6, *V. cholerae* non-O1/non-O139 strain VC-16; 7, negative control; M, 100 bp ladder.

those belonging to serogroups O1 and O139. Similarly, TCP has infrequently been allied with the environmental *V. cholerae* strains (Nair et al., 1988). The major structural subunit of TCP is encoded by the *tcpA* gene, verified in all *V. cholerae* non-O1/non-O139 isolates. Out of the 23 isolates only one strain was positive for *toxR* gene. The regulation and expression of genes for growth and survival depends on the regulon *toxR*. ToxR, a 32-kDa transmembrane protein, is the master regulator, and its expression is dependent upon environmental growth conditions (incubation temperature, pH, osmolarity, bile salts, oxygen tension, hydrostatic pressure, and amino acid composition of the medium) (DiRita 1992). The *toxR* gene encodes a transcriptional activator controlling CT gene expression (*ctxA*), TCP biogenesis (*tcpA*), outer membrane protein expression (*ompU*), and at least 17 distinct genes in O139 and O1 strains (Miller et al., 1987). Genes encoding TCP had been suggested to be part of a large genetic element consisting of a cluster of genes; we looked for the presence of the *tcpA* gene. In this study the *tcpA* El Tor gene was presented in one strain of *V. cholerae* non-O1/non-O139 in agreement with previous report *tcpA* El Tor gene was also found in 9 of 39 non-O1/non-O139 Brazilian strains (Chakraborty et al., 2000; Nandi et al., 2000). These results suggest that this strain may have a selective advantage over nonpathogenic strains, with an ability to colonize the human intestine, and become toxigenic (Karaolis et al., 1998).

In the study reported here, we observed a low frequency of the *toxR* genes which regulated the expression of other virulence genes in *V. cholerae* depending on the source or ecosystem (seawater, seafood, wastewater, and clinical specimens). Aquatic and marine ecosystems are subjected to large spatial and temporal nutrient fluxes

arising from seasonal and geographic variations in temperature, salinity, nutrient input, pH, oxygen tension, etc. (Roszak et al., 1987). The mechanisms by which environmental conditions affect the coordinated expression of virulence factors by *V. cholerae* remain poorly understood. Hase and Mekalanos (1998) proposed a model where *toxR* is involved in sensing various environmental and internal stimuli and are required for the production of TCP in *V. cholerae*. The *toxR* gene was shown to be involved in the regulation and expression of several genes of *V. cholerae* (Ottemann et al., 1994). Subsequent studies had demonstrated the presence of *toxR*-related gene sequences in other organisms belonging to *Vibrio* spp., although their sequences showed considerable variations (Osorio et al., 2000). As a matter of fact, the *toxR* gene was recently used as a probe for the species-specific identification of *Vibrio parahaemolyticus* (Kim et al., 1999). Interestingly, this gene probe developed for *V. parahaemolyticus* failed to detect *V. cholerae* despite 52% identity in their *toxR* gene sequences. These results appeared to be somewhat consistent with our data since the *toxR* probe for *V. cholerae* recognized only one strain of 23 isolates.

Non-O1/ non-O139 *V. cholerae* strains can no longer be ignored (Rivera et al., 2001). The rationale for continuous monitoring is based first on the emergence of serogroup O139 (Bengal) in Bangladesh (CT positive) and Argentina (CT negative), each of which clearly evolved independently (Stroeher et al., 1997). The sixth pandemic, the seventh pandemic, and U.S. Gulf Coast isolates represent three different clones, each independently evolved from environmental non-O1 *V. cholerae* isolates (Karaolis et al., 1998). Other epidemic serogroups had emerged, including *V. cholerae* O31

(Morris et al., 1990), O37 (Dakin et al., 1994), O53 (Mukhopadhyay et al., 1995), O10, and O12 (Dalsgaard et al., 1995; Sharma et al., 1997). The emergence of a new clone of the *V. cholerae* O1 El Tor in Calcutta, India (Sharma et al., 1997), and in Bangladesh (Faruque et al., 1998) has been reported. Its possibility exists that additional new strains of toxigenic *V. cholerae* with epidemic potential may emerge in the future.

The results of the present study parallels those of a previous study using a high-resolution DNA fingerprinting method to show that clinical toxigenic *V. cholerae* isolates are closely related to the so-called non-toxigenic environmental strains (Jiang et al., 2000) and further suggests that the toxin genes are mobile among environmental isolates. However, it is still not clear how these genes spread in an aquatic environment in an area of non-epidemic. It is possible that a different mechanism of gene transfer operates for *V. cholerae* in aquatic environments. It is presently unclear whether CT and TCP genes among the environmental isolates are expressed or what their ecological and biological function is in the aquatic environment. It has been reported that non-cholera-toxin-producing *V. cholerae* non-O1, non-O139 strains possess *toxR* gene, and can acquire the *tcp* gene from toxigenic *V. cholerae* O1 by horizontal gene transfer (Karaolis et al., 1999) when they are exposed to the filamentous bacteriophage VPI Φ . The *ctx* element can be acquired by exposure to the toxiniferous phage CTX Φ (Faruque et al., 1998; Waldor and Mekalanos 1996).

In this study, we report presence of *Vibrio cholerae* non-O1/non-O139 *ctxA* negative and for *tcpA* El Tor, *toxR* and *O1rfb* positive in small numbers in the Arabian Gulf coastal water environment. While these strains did not cause epidemics, there are environmental factors that may change, enhance multiplication or dominance, or a selection *V. cholerae* genotypes, and the strains themselves need to be tested for potential selective advantage under selected environmental conditions.

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