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Comparison of Distribution of Virulence Determinants in Clinical and Environmental Isolates of *Vibrio cholera*

Bita Bakhshi[1 & 2], Mohammad Reza Pourshafie[*1], Farahtaj Navabakbar[2], Akbar Tavakoli[2] Fereshteh Shahcheraghi[1], Mansoor Salehi[2], Ziba Faradjzadegan[2] and Seyed Mohsen Zahraei[3]

[1]Dept. of Bacteriology, Pasteur Institute of Iran, Pasteur Ave., Tehran; [2]Faculty of Medicine, Isfahan University of Medical Sciences, Hezarjerib Ave., Isfahan; [3]Dept. of Enteric Bacteria, [3]Center for Disease Control, Tehran, Iran

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ABSTRACT

Background: The virulence of a pathogenic *Vibrio cholerae* is dependent on a discrete set of genetic determinants. In this study, we determined the distribution of virulence determinants among the clinical and environmental isolates of *V. cholerae*. **Methods:** The antibiotic resistance profiles of the isolates were determined using standard disk diffusion assay. PCR assay was performed to analyze the presence of toxin genes of *ctx*, *zot* and *ace*. The composition of cholera toxin encoding element (CTX) region flanking of the *V. cholerae* isolates was also analyzed. **Results:** All of the clinical isolates (100%) showed a complete set of virulence genes and also the attachment site of the filamentous bacteriophage CTXϕ. None of the environmental isolates contained the virulence genes and the attachment site of the CTXϕ. Analysis of the flanking regions including the toxin-linked cryptic element and repeat in toxin genes revealed their integrity in the clinical isolates while in the environmental isolates they were absent or contained incomplete sequences. Comparison of the antibiotic resistance assay of the environmental and clinical isolates showed a significant difference in the resistance profiles of the isolates obtained from the two sites. High rates of resistance to co-trimoxosol, streptomycin and chloramphenicol were found with clinical isolates. **Conclusion:** The absence of all virulence determinants in the environmental strains may suggest that certain ecological features must be present for *V. cholerae* to acquire a complete set of virulence determinants and to turn them into pathogenic strains. *Iran. Biomed. J. 12 (3): 159-165, 2008*

Keywords: *Vibrio cholerae*, Virulence determinants, Environment

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INTRODUCTION

Vibrio cholerae includes both pathogenic and non-pathogenic strains that vary in their virulence gene contents [1-3]. The pathogenic and epidemic strains of *V. cholerae* possess two essential genetic elements (i) cholera toxin encoding element (CTX) element which is part of the filamentous bacteriophage (CTXϕ) genome [4] involved in coding for cholera toxin (CT) as the most important virulence factor of *V. cholerae*, and (ii) the *V. cholerae* pathogenicity island which encodes the toxin co-regulated pilus (TCP). The TCP is the adhesin that is coordinately regulated with CT production which functions as an essential colonization factor in the gut as well as the receptor for CTXϕ [5]. Upstream of the CTX genetic element is toxin-linked cryptic (TLC) element and 3' of the CTXϕ contains a region which encodes repeat in toxin (RTX) gene cluster composed of the toxin (*rtxA*), activator (*rtxC*) and transporters (*rtxBD)* [6-8]. It is presumed that CT and TCP elements are exclusively associated with the clinical strains of *V. cholerae* serogroups O1 and O139. Nevertheless, a few reports on the presence of CT among the environmental strains of *V. cholerae* have been published [9]. TCP has also been shown to be associated with clinical *V. cholerae* O1 and O139 and it is rarely found in the environmental isolates. For example, the presence of *tcp*A of the TCP gene cluster has been reported in non-O1 toxigenic [10] and nontoxigenic non-O1 non-O139 strains [11]. It has been shown by some investigators [8, 12] that the conversion of non-toxigenic environmental

[*]Corresponding Author; Tel. (+98-21) 6640 5535; E-mail: pour@pasteur.ac.ir

strains into epidemic strains *V. cholerae* is possible if they acquire the appropriate set of virulence genes

The aim of this study was to understand the epidemiology and origin of pathogenic strains by determining the prevalence of virulence genes in the clinical and environmental strains of *V. cholerae* isolated in Iran.

MATERIALS AND METHODS

Collecting and processing of environmental samples. Water samples were collected from surface water located at 6 different locations in Tehran (Iran) including Aminabad, 17-E-Shahrivar, Firoozabad, Beheshti, Yakhchiabad and Afsarieh lakes. The samples were filtered using Whatman no.1 filter paper and subsequently filtered through a 0.45-µm- pore-size membrane by using vacuum pressure of 15 to 20 lb/in^2. The membrane was cut into eight pieces and vortexed in a 2-ml of 10 mM PBS (pH 7.4) for 3 min. One milliliter of the suspension was added to 10 ml of alkaline peptone water (APW) containing peptone (1%, wt/vol) and NaCl (1%, wt/vol) (pH 8.5) for enrichment with shaking (100 rpm) at 37°C for 16-18 h. Each sample was streaked on thiosulfate-citrate-bile-sucrose agar plates and incubated at 37°C overnight [5]. The resulting yellow colonies were further examined. The identity of *V. cholerae* strains was defined by 11 biochemical assays including colony morphology, oxidase, motility, sucrose fermentation, lactose fermentation, growth in 0% NaCl, arginine dehydrolase, ornithine decarboxylase, methyl red, voges-proskauer and indole test [13, 14] and confirmed by PCR amplification of 16s-23s rRNA intergenic region specific for *V. cholerae* [15]. The *V. cholerae* strains (*n* = 25) were isolated and subjected to further analysis.

Clinical strains. *V. cholerae* strains (n = 25) of clinical origin, collected from four different provinces in Iran (Tehran, Qom, Golestan and Zahedan) in 2005, were included in this study. The specimens were collected on the sterile swabs and were enriched in APW. The suspected samples were then placed in Carry-Blair transport and shipped to the Department of Bacteriology, Pasteur Institute of Iran (Tehran). Biochemical assays were performed to confirm the identity of *V. cholerae* strains. The isolates were identified as described in the above section.

Serology. In this study, serogrouping of all isolates was performed with polyvalent O1 and monospecific Inaba and Ogawa antisera (Mast Diagnostics Ltd., Bootle, Mersey side, UK).

Anti-microbial susceptibility testing. Antibiotic susceptibility patterns were tested by the standard disk diffusion technique according to NCCLS (National Committee for Clinical Laboratory Standards, 2001) guidelines with the following antibiotics: gentamicin (10 µg), doxycycline (30 µg), oxytetracycline (30 µg) and tetracycline (30 µg) were purchased from Difco laboratories (Detroit, MI, USA) and ciprofloxacin (5 µg), streptomycin (5 µg), ampicillin (10 µg), polymixin B (300 U), chloramphenicol (30 µg) and co-trimaxazole (25 µg) were purchased from Becton Dickinson and Company (Sparks, MD, USA).

DNA extraction and PCR assay. The chromosomal DNA from the isolates was obtained by a simplified method whereby one isolated colony was suspended into 200 µl of sterile distilled water and boiled for 5 min and 5 µl of supernatant was used as the template in a PCR reaction [16]. PCR assay was performed using primers specific for the toxin genes inside the CTX genetic element including *ctx, zot* and *ace* [17-19]. The CTXϕ attachment site was also investigated in the two groups of strains using primers attRS-F or attRS-R specific for 18 nucleotide attachment site with any of the forward or reverse primers inside the core or RS1 regions (data not shown) [20]. The flanking regions including RTX cluster (1448-F, 1448-R and 1451-F, 1451-R) and TLC element (1465-F, 1465-R and 1469-F, 1469-R) were also subjected to analysis due to the important role which may have played in the acquisition of the toxin genes and evolution of the pathogenic strains. The DNA was extracted and PCR was performed in a 25-µl total reaction volume containing 20 µl sterile water, 2.5 µl 10× *Taq* polymerase buffer, 0.6 µl $MgCl_2$ (25 mM), 0.3 µl dNTPs (10 mM), 0.5 unit of *Taq* DNA polymerase, 25 pmol of each primer. The cycling conditions were as follows: preincubation at 94ºC for 5 min, 30 cycles of 1 min at 94ºC for denaturation, 1 min at 58ºC for annealing, 2 min at 72ºC for elongation and incubation at 72ºC for 3 min for final elongation. Primer designation and their sequence used in this study are depicted in Table 1. *V. cholerae* ATCC 14035 was used as positive control in each PCR assay [21].

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Table 1. Primers used in this study.

Designation	Sequence			Reference
prVC-F	5'	AGTCACTTAACCATTCAACCCG	3'	15
prVCM-R	5'	TTAAGCGTTTTCGCTGAGAATG	3'	
ctx-F	5'	CGGGCAGATTCTAGACCTCCT	3'	18
ctx-R	5'	CGATGATCTTGGAGCATTCCCAC	3'	
zot-F	5'	TGGCTTCGTCTGCTGCCGGCGATT	3'	19
zot-R	5'	CACTTCTACCCACAGCGCTTGCGC	3'	
ace-F	5'	TAAGGATGTGCTTATGATGGACACCC	3'	17
ace-R	5'	CGTGATGAATAAAGATACTCATAG	3'	
VC1448-F	5'	TGCTTCATCCAAAATCAGCA	3'	This study*
VC1448-R	5'	TCATCAGCGGTAATCGAGAA	3'	This study
VC1451-F	5'	TGTTCGGCGATAACATTCAG	3'	This study
VC1451-R	5'	TTTGTGAACCACGTCTGACCCTT	3'	This study
VC1465-F	5'	CTTTGGCCGTGTCTATTGGT	3'	This study
VC1465-R	5'	TAAATACGTGCGGCTCAACA	3'	This study
VC1469-F	5'	AACTCATGAGCAAGGCGTTT	3'	This study
VC1469-R	5'	GCTCGGAAGACTTTCGCTTA	3'	This study
attRS-F	5'	CCTTAGTGCGTATTATGT	3'	16
attRS-R	5'	ACATAATACGCACTAAGG	3'	

*primers designed according to the *V. cholerae* strain N16961 whose genome is fully sequenced and is available on BLAST (*www.ncbi.nlm.nih.gov*).

RESULTS

Identification of isolates and serology. A total of 50 *V. cholerae* strains including 25 clinical and 25 environmental strains were isolated and analyzed in this study. All of the clinical isolates were O1 Inaba and all of the environmental isolates were non O1-non O139 serotypes (Table 2).

Isolates and antibiotic susceptibility. All 50 isolates including 25 clinical and 25 environmental strains were identified as *V. cholerae* O1 serotype Inaba for clinical and non O1-non O139 serotype for environmental isolates. Antibiotic susceptibility testing of isolates showed 12 and 10 antibiotic resistance patterns among the clinical and environmental isolates, respectively. Pattern one, SXT^r, S^r, CHL^r, TE^r, PB^r, with 24% was the predominant pattern among the clinical isolates while the predominant pattern among the environmental isolates was 36% which represents no resistance to any of the antibiotics examined. The rate of antibiotic resistance among the clinical *V. cholerae* strains was significantly higher as compared with the environmental strains. The most noticeable difference was seen for the SXT, streptomycin and chloramphenicol. Resistance to these three antibiotics was observed in 92%, 92% and 88% of the clinical and 16%, 12% and 8% of environmental isolates, respectively. No resistance was seen to gentamicin and ciprofloxacin in the clinical or environmental isolates.

PCR assay. PCR revealed the presence of *ctx*, *zot*, and *ace* genes in 100% of clinical isolates. None of the environmental strains contained these toxin genes. Analysis for the presence of attachment sites and the flanking gene clusters revealed that the

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Table 2. A summary of characteristics of *V. cholerae* strains of clinical and environmental origin.

Source	Serology	*ctx*	*ace*	*zot*	*VC1448*	*VC1451*	*VC1465*	*VC1469*	percent
Clinical	O1	+	+	+	+	+	+	+	100
Environmental	nonO1-nonO139	-	-	-	+	+	-	-	64
Environmental	nonO1-nonO139	-	-	-	+	+	+	-	24
Environmental	nonO1-nonO139	-	-	-	-	+	+	-	4
Environmental	nonO1-nonO139	-	-	-	+	-	-	-	4
Environmental	nonO1-nonO139	-	-	-	-	-	+	-	4

clinical isolates possessed 18 nucleotide attachment sites while none of the environmental isolates contained this nucleotide sequence. The chromosomal RTX cluster genes with VC1448 and VC1451 ORF existed in 100% and 100% of clinical and 92% and 92% of the environmental isolates for both of these genes. The VC1465 and VC1469 ORF in the TLC element were present in 100% and 100% of the clinical and 32% and 0% of environmental isolates, respectively (Table 2). Representatives of PCR products are shown in Figures 1 and 2.

DISCUSSION

In this study, the occurrence and distribution of the selected virulence genes in the clinical and environmental strains of *V. cholerae* isolated in Tehran, Iran, were examined. The clinical strains were *V. cholerae* O1 Inaba while environmental strains were non-O1, non-O139 serotype. None of the environmental strains carried the toxin genes whereas all clinical isolates harbored all toxin genes: *ace, zot* and *ctx* (Table 2).

Chakraborty and colleagues [5] have reported that 79% of environmental non-O1, non-O139 serotype, isolates didn't posses any of the virulence determinants in the CTX genetic element. Moreover, Brazil and colleagues [11] have shown that none of the *V. cholerae* environmental isolates, non-O1 serotype contained *ctx*, *zot* and *ace* genes. Singh and colleagues [22] also have shown the presence of virulence genes including *ctx*, *zot* and *ace* in the clinical and not the environmental isolates of *V. cholerae*, although reports on clinical nonO1-nonO139 strains [23] and also the presence of virulence genes including ctxAB, in some non-O1, non-O139 serogroups [5] should not be ignored.

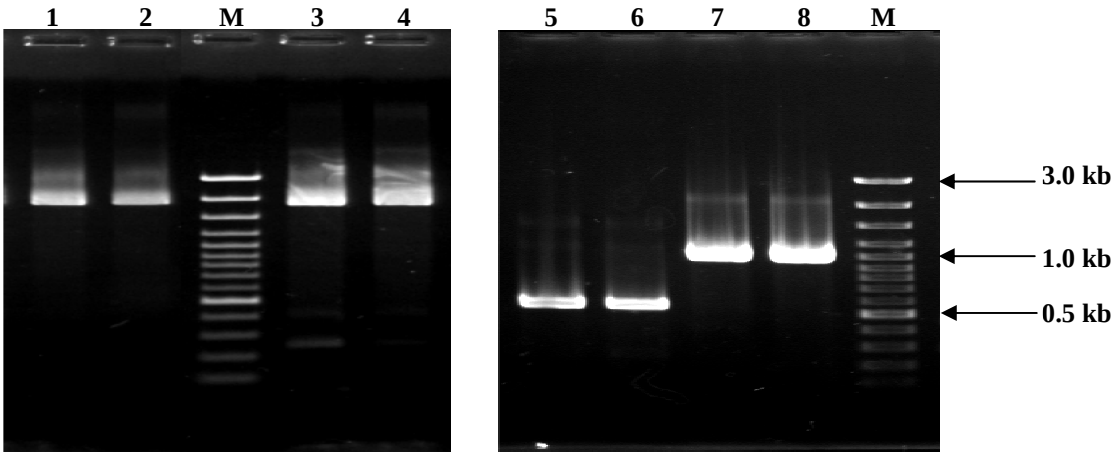


Fig. 1. Representatives of amplified DNA products of the CTX flanking regions. Lane 1 and 2, VC1448; lane 3 and 4, VC1451; lane 5 and 6, VC1465; lane 7 and 8, VC1469.

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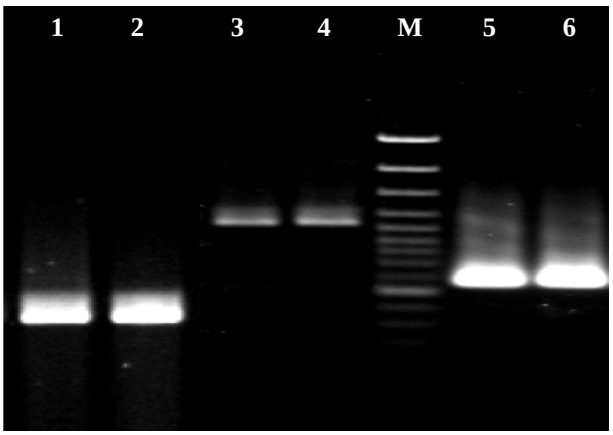


Fig. 2. Representatives of amplified DNA products of the genes inside the CTX element. Lane 1 and 2, ace-F/R; lane 3 and 4, zot-F/R,; lanes 5 and 6, ctx-F/R.

The CTX attachment site is a region comprises an 18-bp conserved region adjacent to the CTX element. All clinical strains were shown to carry this region while none of the environmental isolates contained this genetic region. The presence of this site is necessary for the attachment of CTXϕ phage into the genome of *V. cholerae* [20]. On the contrary, the absence of this attachment site in the environmental isolates has raised the question of whether the defects in the flanking regions could contribute in hindering the isolates for acquisition of the genes involved in the attachment sites and CTX element. The DNA from *V. cholerae* isolates was subjected to PCR analysis for investigation of flanking regions of the CTX element. The RTX gene cluster which lies at 3' end of the CTX element is a chromosomal cluster and presents in pathogenic and non-pathogenic strains of *V. cholerae* [6, 7]. The presence of VC1448 and VC1451 ORF in the majority of the environmental (92%) and clinical (100%) isolates showed that RTX coding cluster was widely distributed in the genome of both pathogenic and non-pathogenic *V. cholerae* isolates (Table 2), and no significant difference in the presence of this cluster among the two groups can be drown ($P>0.05$).

The RTX toxins represent a family of important virulence factors that have disseminated widely among Gram-negative bacteria [5], and its presence in most of the *V. cholerae* isolates would raise the question of whether this toxin gene cluster could be transferred into *V. cholerae* by other Gram-negative bacteria.

A development in the evolution of pathogenic *V. cholerae* strains in obtaining the CTX genetic element is the acquisition of upstream TLC element. It has been suggested that this element could be inserted into the bacterial genome by another bacteriophage [24]. The *V. cholerae* strains which contain CTX element also harbors TLC element simultaneously. On the other hand, the strains which carry TLC element may not harbor the CTX genetic element [24]. This notion is supported by our data that the all toxigenic clinical isolates also carried the two ORF in the TLC element namely VC1465 and VC1469. Moreover, the two ORF were present in 32% and 0% of the environmental isolates, respectively, with significant difference between the two clinical and environmental groups ($P< 0.05$).

The reason for the less number of environmental isolates to contain the TLC elements could be because of their bacteriophage originality as opposed to the RTX gene cluster which is found in abundance in both environmental and clinical isolates.

Antibiotic resistant pattern was also included in the comparison of the virulence determinants of clinical and environmental isolates. This comparison is summarized in Tables 3 and 4 depicting the significant difference in the resistance patterns of the isolates to antibiotics including SXT, streptomycin and chloramphenicol ($P< 0.05$). The resistance to these antibiotics has been shown to be encoded by the genes in the conjugative SXT transposon. It is, therefore, possible that such transposons were extensively disseminated in the clinical but not environmental isolates.

In conclusion, results of this study shows that clinical and environmental isolates of *V. cholerae* differ in virulence and resistance encoding determinants, in addition to the serogroups and phenotypic characteristics. These differences may show the evolutionary status of non-toxigenic environmental isolates of *V. cholerae* to emerge into pathogenic strains by acquisition of virulence associated genes which in turn, mandates the need to monitor the occurrence, stability and evolution of *V. cholerae* strains in the environment.

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Table 3. Percentage of antibiotic resistance patterns among the clinical isolates of *V. cholerae*.

Pattern	Percent	SXT	S	CHL	TE	PB	DO	AP	T	CIP	GM
1	24	R	R	R	R	R	S	R	S	S	S
2	16	R	R	R	R	R	S	R	S	S	S
3	12	R	R	R	S	R	S	S	S	S	S
4	8	S	S	S	R	R	S	S	S	S	S
5	8	R	R	R	R	S	S	S	S	S	S
6	8	R	R	R	R	R	S	S	S	S	S
7	4	R	R	R	R	R	R	R	R	S	S
8	4	R	R	R	R	R	R	R	S	S	S
9	4	R	R	R	R	S	S	S	S	S	S
10	4	R	R	R	S	R	S	R	S	S	S
11	4	R	R	R	S	S	S	S	S	S	S
12	4	R	R	S	R	R	S	S	S	S	S
Percentage of resistance		92%	92%	88%	80%	84%	8%	52%	4%	0%	0%

SXT, co-trimoxazole; S, streptomycin; C, chloramphenicol; TE, tetracycline; PB, polymixin B; DO, doxycycline; AP, ampicillin; T, oxytetracycline; CIP, ciprofloxacin and GM, gentamicin.

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Table 4. Percentage of antibiotic resistance patterns among the environmental isolates of *V. cholerae*.

Pattern	Percent	SXT	S	CHL	TE	PB	DO	AP	T	CIP	GM
1	36	S	S	S	S	S	S	S	S	S	S
2	28	S	S	S	S	R	S	S	S	S	S
3	8	R	S	S	S	R	S	S	S	S	S
4	4	R	S	S	S	S	S	R	S	S	S
5	4	R	R	R	R	R	R	R	R	S	S
6	4	S	S	R	R	R	R	R	R	S	S
7	4	S	S	S	R	S	R	S	R	S	S
8	4	S	R	S	S	S	S	R	S	S	S
9	4	S	S	S	S	S	S	R	S	S	S
10	4	S	R	S	S	S	S	S	S	S	S
Percentage of resistance		16%	12%	8%	12%	44%	12%	16%	12%	0%	0%

SXT, co-trimoxazole; S, streptomycin; C, chloramphenicol; TE, tetracycline; PB, polymixin B; DO, doxycycline; AP, ampicillin; T, oxytetracycline; CIP, ciprofloxacin and GM, gentamicin.

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