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The role of CTX and RS1 satellite phages genomic arrangement in *Vibrio cholera* toxin production in two recent cholera outbreaks (2012 and 2013) in IR Iran

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ABSTRACT

The objective of the present study was to investigate the genomic arrangement of CTX/RS1 prophages in 30 *Vibrio cholerae* strains obtained from 2 consecutive years of cholera outbreak and to compare the role of different CTX/RS1 arrangements in cholera toxin expression among the El Tor strains.

Profile A with TLC-RS1-CTX-RTX arrangement was observed in 46.7% of the isolates with RS1 phage locating adjacent to TLC element. About 50% of the isolates showed Profile B with TLC-CTX-RS1-RTX arrangement and one single isolate (3.3%) revealed TLC-CTX-RS1-RS1-RTX arrangement (Profile C). No RS1 element was detected to be adjacent to TLC element in B and C profiles. No truncated CTX phage genome was detected among the isolates of 2 years.

Different CTX-RS1 arrangement profiles (A, B, and C) with different RS1 copy numbers and locations uniformly showed low level of cholera toxin production in El Tor strains with no significant difference, revealing that different RS1 copy numbers and locations have no effect on cholera toxin production level (p-value >0.05). However, increased cholera toxin expression was observed for control *V. cholerae* classical biotype strain.

In conclusion, variations in RS1 prophage did not affect CT expression level in related El Tor *V. cholerae* strains. CTX genotyping establishes a more valuable database for epidemiologic, pathogenesis, and source tracking purposes.

1. Background

Vibrio cholerae is a gram-negative bacterium causing severe watery diarrhea. Two serogroups of O1 and O139 belonging to this pathogen are known as the most common agents of epidemic and pandemic cholera [1,2]. Many molecular typing methods are used to assess the clonal relationship and virulence properties of clinical and environmental *V. cholerae* [3,4]. Cholera toxin is the major virulence factor which is encoded by *ctxAB* gene carried by a filamentous bacteriophage (CTX phage). Previous studies showed that CTX bacteriophage is composed of a 4.5 kb core region containing genes encoding cholera toxin (*ctxAB*) and multiple virulence factors like *zot, ace, cep, orfU*. The core region is flanked by a 2.4 kb sequence known as RS2. The arrangement and number of CTX prophages differ in El Tor and classical biotypes; in El Tor strains, multiple copies of CTX prophages can be tandemly arranged on the same chromosome while in classical strains, only one copy of CTX element can be carried on each one of the two chromosomes [5,6]. In El Tor biotype, the CTX element is located between toxin-linked cryptic (TLC) and RTX elements [7].

In EL tor biotype and O139 serogroup, a genetic element known as RS1 is located beside to CTX phage. RS1 is similar to RS2 region with an additional gene called *rstC* [7–9]. Variations in this genetic element cause different genetic arrangements of CTX prophage in *V. cholerae* genome. Different molecular techniques are used to find genetic variations of CTX bacteriophage in *V. cholerae* genome [10].

The expression level of cholera toxin, and subsequently, the severity of the disease are supposed to be affected by CTX element copy number, but the impact of RS1 location and copy number on cholera toxin production is still under way to be understood [11].

The objective of the present study was to investigate the diversity and genetic arrangement of CTX/RS1 elements in 30 *V. cholerae* strains obtained from Iran during a 2-year period (2012

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and 2013 years) and to compare the level of CT expression among El Tor strains with different CTX/RS1 arrangements.

2. Materials and methods

2.1. Ethics approval and consent to participate

The study was reviewed and approved by Medical Ethics Committee of Tarbiat Modares University (Code: IR.MODARES.REC) before the study began (Research Ethical Code: 52/8193).

2.2. Bacterial strains used in this study

A total of 30 *V. cholerae* strains isolated from cholera patients of 2012 and 2013 outbreaks in Iran were included in this study. The identity of strains was confirmed by biochemical and molecular methods [12,13]. Isolates serotyping was performed by polyclonal O1 and monospecific Ogawa and Inaba antisera (Mast Diagnostics Ltd., Bootle, Merseyside, UK), and biotyping was performed by Voges-Proskauer (VP) test. *V. cholerae* ATCC 14035 was used as a positive control in each assay.

2.3. CTX and RS1 satellite phage related genes and TLC and RTX clusters

The presence of CTX cluster related genes was detected by PCR method using primers which specifically amplify *ace*, *zot*, and *ctx* genes. The presence of RS1 satellite phage was detected by *rstC* gene which is supposed to be exclusively present in RS1 phage [14]. TCL and RTX gene clusters were detected by PCR assay for VC1465 (TLC element) and VCVC 1448 (RTX cluster). Primer sequences used in this study are shown in Table 1.

2.4. Copy number of CTX and RS1 satellite phages by southern blot analysis

DNA was extracted from bacterial pellet obtained from overnight culture of bacteria on Brain-Heart Infusion agar, followed by centrifugation. Phenol /chloroform/isoamyl alcohole method was used for DNA extraction, followed by DNA digestion with *pst1* restriction enzyme (Roche) which has only one restriction site within the CTX phage genome [15]. Separation of fragments was performed by overnight electrophoresis of products under low voltage. The DNA fragments were transferred to positively charged nylon membranes (Roche) after alkaline denaturation. A DIG DNA Labelling and Detection Kit (Roche) was used to produce digoxigenin-labelled *ctxA* and *zot* gene probes. The membranes were then hybridized with freshly denatured probes after a pre-hybridization step in hybridization buffer containing 5×SSC (20×SSC is 3M NaCl, 0.3M sodium citrate), 2% w/v blocking reagent (Roche), 0.1% w/v *N*-laurylsarcosine, and 0.02% w/v SDS. Four washing steps were performed, twice in 2×SSC and SDS 0.1% w/v (5 min RT) and twice in 0.5×SSC and SDS 0.1% w/v (15 min 68 °C), to increase the specificity of hybridization and remove the background. Detection was performed by anti-digoxigenin antibody according to the manufacturer's instructions.

2.5. Determination of RS1 satellite phage location

Location of RS1 to be adjacent to TLC element was detected using 2 separate PCR assays. In one assay, forward primer was selected within the VC1465 of TLC element, and reverse primer was chosen to anneal within the rstC gene of RS1 phage. In the second assay, forward primer was complementary to *rstC* gene of RS1 phage in opposite direction, and the reverse primer was paired with VC1448 of RTX cluster.

2.6. Quantification of cholera toxin expression

2.6.1. Reverse transcriptase PCR

A total of 2×10^8 cfu.mL^{-1} of each isolate was used for RNA extraction using RNA extraction kit (sinaClon Bio Science Co, Tehran, Iran), followed by cDNA synthesize 2-step RT-PCR kit (Vivantis, Germany). The housekeeping *recA* gene was used as internal control.

2.6.2. Real-time PCR (RT-qPCR)

The isolates were screened for *recA* and *ctxB* genes, yielding 106 and 46bp bands, respectively. Preparation of cDNA was quantified using SYBR®Premix Ex Taq™ (Takara Bio inc, Shiga, Japan). The SYBR green Premix EX Taq mixure was in a total volume of 20 μL containing 0.8 μL of each primer pair, 2 μL of cDNA sample, and 6.4 μL of distilled water. RT-q PCR program was as follows: one cycle of 95 °C for 5 min as initial denaturation, 40 cycles consisting of 94 °C for 15 s, 60 °C for 1min and 68 °C for 1min. Distilled water was included in each round as negative control. Each RT-qPCR reaction was performed in triplicates. Classical *V. cholerae* O1 ATCC 14035 was used as a standard control. The $2^{-\Delta CT}$ was used for calculation of *ctxB* relative expression in isolates under study.

Table 1
Primers used in this study.

Primer Name	Sequence (5′ to 3′)	Amplicon Size (bp)	Application	Reference
RS1(rstC)	CGTTCAGGCGCTTATACAGAC CAGTGATGGCTCAGTCAATGC	147bp	PCR	This study
Zot	TGGCTTCGTCTGCTGCCGGCGATT CACTTCTACCCACAGCGCTTGCGC	1083bp	PCR/Probe	[20]
Ace	TAAGGATGTGCTTATGATGGACCC CGTGATGAATAAAGATACTCATAG	316bp	PCR	[21]
VC1465-F VC1465-R	CTTTGGCCGTGTCTATTGGT TAAATACGTGCGGCTCAACA	545bp	PCR	[10]
VC1448-F *VC1448*-R	TGCTTCATCCAAAATCAGCA TCATCAGCGGTAATCGAGAA	1805bp	PCR	[10]
ctxB	GGTTGCTTCTCATCATCGAACCAC GATACACATAATAGAATTAAGGAT	460bp	PCR/Probe Real time PCR	[10]
recA	ATTGAAGGCGAAATGGGCGATAG TACACATACAGTTGGATTGCTTGAG	115bp	PCR	[11]