Quantitative expression of cholera toxin mRNA in *Vibrio cholerae* isolates with different CTX cassette arrangements

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Cholera toxin (CT) is the major virulence factor produced by *Vibrio cholerae*. Several genomic arrangements within the CTX cassette have been elucidated in *V. cholerae*. Previously, it was shown that three different CTX cassette arrangements, one complete CTX cassette (arrangement A), one complete and two incomplete CTX cassettes (arrangement B), and two complete CTX cassettes (arrangement C), exist within *V. cholerae* isolates. In the present study, the level of CT expression by *V. cholerae* isolates carrying different CTX arrangements was evaluated. Real-time quantitative PCR analysis showed unequal production of CT mRNA in *V. cholerae* isolates with different CTX arrangements. *V. cholerae* with the CTX arrangement C expressed more CT mRNA than isolates with the other CTX arrangements. In addition, CT mRNA was expressed more in the isolates with CTX arrangement B than in those with arrangement A. Overall, these results suggest that the arrangement and number of regulatory elements (*rstA*) within the CTX cassette could affect the level of expression of CT.

INTRODUCTION

Cholera remains a major health problem in developing and underdeveloped countries. Today, we are facing the continuation of the seventh cholera pandemic, which has been spread from South-east Asia across the Middle East into Central America and Africa (Pourshafie et al., 2000). Cholera toxin (CT) is a potent enterotoxin that plays a major role in the pathogenesis of *Vibrio cholerae* through a complex cooperation with CTXΦ phage and other phages (Bakhshi et al., 2008). Environmental factors also affect expression of CT through a number of regulatory genes such as tcpP, tcpH and toxT. ToxT attaches to the ToxT-binding motif toxbox within the CTX cassette, located upstream of the ctxAB genes, which encode CT (Withey & DiRita, 2006). The production of ToxT protein is also affected by the products of the toxR gene and the quorum-sensing system (Klose, 2001; Tsou et al., 2009). The RS1 element, containing *rstA*, *rstB*, *rstR* and *rstC*, is located upstream or downstream adjacent to the CTX cassette (Davis & Waldor, 2003). These genes encode proteins for replication and activation (*RstA*), integration (*RstB*), regulation of gene expression (*RstR*) and anti-repression (*RstC*). It has been shown that RS1 has a positive effect on the *ctxAB* genes, resulting in increased production of CT by modulation of the RS1 genomic segment (Davis & Waldor, 2003). A previous study showed that each CTX cassette contains an RS2 element and *ctxAB* genes. The RS2 element is like RS1 but lacks the *rstC* ORF; thus, the CTX cassette contains only the *rstA*, *rstB* and *rstR* ORFs (Davis & Waldor, 2003). A previous report by this laboratory showed that, among *V. cholerae* isolates obtained from various parts of Iran, three distinct CTX cassettes with different arrangements existed (Bakhshi et al., 2008) (Fig. 1).

We showed that incomplete CTX cassettes contained all elements of the CTX cassette except the *ctxAB* genes. The present study was designed to determine the level of CT mRNA production in *V. cholerae* isolates with different CTX arrangements and to explore potential associations of specific arrangements and levels of CT production.

Abbreviations: CT, cholera toxin; C<sub>T</sub>, cycle threshold; qPCR, real-time quantitative PCR; RT-PCR, reverse transcriptase PCR.
**METHODS**

**Bacterial strains and growth conditions.** In total, 94 clinical isolates of *V. cholerae* O1 El Tor orphaned from different outbreaks between 2004 and 2009 were studied. A previous study in this laboratory showed that these isolates were Inaba and Ogawa serotypes (Bakhshi *et al.*, 2008). In the present study, three strains were selected randomly, one from each of the three different arrangements of the CTX cassette. All strains were cultured in synthetic AKI media with shaking and aeration for 15–18 h. The synthetic AKI medium was prepared according to a protocol described previously (Iwanaga & Kuyyakanond, 1987).

**RT-PCR.** For each isolate, $2 \times 10^9$ c.f.u. ml$^{-1}$ was used for total RNA extraction using a RNAeasy Protect Bacteria Mini kit (Qiagen). Separately, an equivalent concentration of total RNA was selected from each sample as template for RT-PCR. cDNA synthesis and PCR amplification were performed using a QuantiTect reverse transcription kit (Qiagen). RT-PCR was performed in the presence of random amplification were performed using a QuantiTect reverse transcription kit (Qiagen). RT-PCR was performed in the presence of random primers at $42 \degree C$ for 10 min. Amplification of *recA* was used as the internal control. The amplified products were verified by sequencing.

**Real-time quantitative PCR (qPCR).** Prepared cDNA was quantified using SYBR Green 1 dye. The amount of cDNA used for qPCR from each sample was 50 ng ml$^{-1}$. Four primers were designed: *recA*-f (5'-ATTGAGGCCGAAATTGCCGATG-3'), *recA*-r (5'-TACACATTACGTTGGATTGCTTGAG-3'), *ctxAB*-f (5'-TATGCCAAGAGGACAGAGTGGAG-3') and *ctxAB*-r (5'-AACATATCCATCATCGTGCCGTC-3'). In the present study, the *recA* gene was considered a housekeeping gene. A SYBR Green qPCR assay was performed in a 20 μl volume containing 2 × QuantiTect SYBR Green PCR Master Mix (Qiagen), 0.25 μM (each) specific primer set and 2 μl of cDNA sample. Amplification of the primers, data acquisition and relative expression analysis were carried out in a Chromo4 real-time detector (Bio-Rad). qPCRs were performed as follows: one cycle of 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s, and a final extension at 72 °C for 2 min. Following amplification, melting-curve analysis of the PCR products was performed to determine the specificity of the qPCR using genomic DNA for each gene studied to confirm that the primers amplified at the same rate and to validate the experiment (55–95 °C, increasing by 0.2 °C s$^{-1}$).

In the qPCR, a negative control of distilled water was included in each run. The qPCR was performed twice in triplicate. Classical *V. cholerae* O1 ATCC 14035 was used as a standard control.

**RESULTS AND DISCUSSION**

The amplicons obtained for the *recA* (106 bp) and *ctxAB* (115 bp) genes were verified by sequencing. The presence of a single PCR product was confirmed by melting-curve analysis, which resulted in a single product-specific melting curve. PCR efficiencies were determined as between 1.90 and 1.94. The relative expression ratio was calculated for each gene of interest by a mathematical model using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001). This model allows for relative changes in gene expression in qPCR experiments and relates the PCR signal of the target transcript in the treatment to that of the untreated control.

Table 1 shows the results of cycle threshold ($C_t$) analysis for the CTX arrangements A, B and C and the classical strain. The mean ratios were also calculated as 0.05, 0.11 and 0.25 for the CTX arrangements A, B and C and were significantly different from that of the classical strain, which was given a value of 1 ($P<0.05$).

The results showed a significant difference between the clinical isolates, regardless of their arrangement, and the classical strain (ATCC 14035) ($P<0.05$ for each). Kaper *et al.* (1995) previously showed that CT production in the classical strain was significantly higher than that in the El Tor strain of *V. cholerae*. In addition, DiRita *et al.* (1996)

**Table 1.** Cycle threshold ($C_t$) results for the A, B and C arrangements and the classical *V. cholerae* O1 strain ATCC 14035

$\Delta C_t$ was calculated as: $\Delta C_t$ (test)−$\Delta C_t$ (calibrator). Ratio=efficiency$^{-\Delta C_t}$.

<table>
<thead>
<tr>
<th>Arrangement</th>
<th>Mean $C_t$ <em>recA</em> ± sd</th>
<th>Mean $C_t$ <em>ctxAB</em> ± sd</th>
<th>Mean $\Delta C_t$</th>
<th>Mean ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>22.74 ± 0.19</td>
<td>25.88 ± 0.22</td>
<td>4.37</td>
<td>0.05</td>
</tr>
<tr>
<td>B</td>
<td>23.61 ± 0.31</td>
<td>25.78 ± 0.37</td>
<td>3.41</td>
<td>0.11</td>
</tr>
<tr>
<td>C</td>
<td>23.34 ± 0.30</td>
<td>24.26 ± 0.30</td>
<td>2.16</td>
<td>0.25</td>
</tr>
<tr>
<td>Classical</td>
<td>25.58 ± 0.14</td>
<td>24.35 ± 0.39</td>
<td>1.23</td>
<td>1.0</td>
</tr>
</tbody>
</table>
showed that the level of CT in the classical strain was higher than that in the El Tor strain because of the influence of toxR on CT production in the classical strain. Both classical and El Tor strains have been shown to express equivalent levels of ToxR. In contrast, the classical strain expresses more ToxT, which has a higher binding affinity to toxbox, resulting in higher expression of CT (González-Bonilla et al., 1994; DiRita et al., 1996).

In this study, we compared the C_t value of classical V. cholerae with El Tor strains with three different CTX arrangements, A, B and C. The results demonstrated the production of lower levels of CT mRNA in the El Tor strains with the various CTX arrangements. The C_t data also suggested that ctxAB gene expression was approximately fourfold and twofold higher in the CTX arrangement C compared with the arrangements A and B, respectively. The differences between arrangements C and A are: (i) the presence of two copies of the complete CTX in arrangement C, and (ii) the position of RS1 downstream of CTXΦ in arrangement C compared with upstream in arrangement A. The data support the suggestion that the position of RS1 may have a direct effect on expression of the ctxAB genes (Davis & Waldor, 2003). This difference in positioning of RS1 could result in enhanced attachment of the RNA polymerase enzyme leading to higher expression of the ctxAB genes (Faruque et al., 2002, 2003). In contrast, the twofold increase in the production of CT in arrangement C compared with arrangement B could be the result of: (i) the presence of one complete CTX and two incomplete CTX cassettes in arrangement B, and (ii) the upstream positioning of RS1. Arrangement B produced twice as much CT mRNA as arrangement A, which could be explained by the presence of three copies of the rstA gene in arrangement B compared with one in arrangement A.

Although the CTX arrangement (CTX cassette and RS1) in the classical strain was similar to arrangement A of the El Tor strain, the results showed that the mean production of CT mRNA was 1.0 in the classical strain compared with 0.05 in arrangement A of the El Tor strain, indicating 20 times less CT production in the strain with arrangement A. In conclusion, the results from this study suggest that other factors, besides the genomic arrangement within the CTX cassette, modulate the production of CT by regulating the CTX cassette, supporting the idea that CT production in V. cholerae is a multi-factorial phenomenon.

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REFERENCES


