The Liposome-PCR Assay is More Sensitive than the VET-RPLA at Detecting Cholera Toxin in Feces and Water

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ABSTRACT

Practical detection of cholera toxin (CT) by a liposome polymerase chain reaction (LPCR) immunoassay was compared to an established V. cholerae enterotoxin and E. coli heat-labile enterotoxin reversed passive latex agglutination (VET-RPLA) assay. The LPCR and VET-RPLA assays detected CT in the range of 10 pg-100 ng/mL and at least 4-19 ng/mL in simulated feces and environmental water, respectively.
Diarrhea transmitted by the fecal-oral route is a major public health burden in the developing world. The enterotoxin (CT) of *Vibrio cholerae* is a major cause of the diarrhea and dehydration in epidemic diarrhea. Monitoring CT can track cholera outbreaks, control epidemics, protect consumers and patients, and possibly prevent bioterrorism. Direct monitoring of CT is important, because the presence of bacteria, even the O1 and O139 serogroups isolated from cholera epidemics, and the gene encoding CT, fail to define toxigenic *V. cholerae* (12).

CT is frequently identified by ELISA or agglutination immunoassays. We reported highly sensitive (subattomolar) detection of CT by a liposome polymerase chain reaction (LPCR) immunoassay (8,9). LPCR is an in-house assay that was tested under laboratory conditions and is not currently FDA validated (3). The purpose of this study was to examine the performance of LPCR in biologically relevant matrices in comparison to a standard assay for CT. Based upon our literature search, the *V. cholerae* enterotoxin and *E. coli* heat-labile enterotoxin reversed passive latex agglutination (VET-RPLA) kit (Oxoid; Remel, Lenexa, KS; TD0920 A) is the only commercially available test for CT. While a number of highly sensitive CT detection methods have been described, including detection limits of some 600 molecules (4), the required antibodies are not commercially available (1,4,5,11,13 and references therein). The VET-RPLA assay is a good standard because its detection limits (1-2 ng/mL) are essentially the same as can be obtained by modern ELISAs (1,7) and its sensitivity is equal to PCR and classical microbiological methods (5,6).

**Matrices.** De-identified normal human feces was from BioChemed Services (Winchester, VA). To approximate "rice-water" consistency, stool was extracted with 1% casein in PBS (1 mL buffer was added to 1 g stool), clarified, and 0.2 µm filtered. Muddy water was collected from a
local ditch, spiked with 1% v/v 1 M phosphate buffer, pH 7, and treated as above. No nucleic acid extraction was performed. These were considered “100%” solutions.

**Quantification of CT by VET-RPLA** was performed in triplicate according to the manufacturer’s instructions without a 24 hr bacterial amplification. Assay volumes were 25 µL sample plus 25 µL of the manufacturer’s buffer. The highest two-fold dilution that produced positive agglutination in a 24 hr period defined the detection limit. We reproduced the VET-RPLA detection limits (~1 ng/mL) of CT (Sigma; C8052) described by others (1, VET-RPLA package insert). Ten-fold dilutions of CT were prepared in dilutions of water or feces in the manufacturer’s buffer. When dilutions were prepared in 1:2 environmental water, the detection limit was ~1 ng/mL (Table 1). When dilutions were prepared from feces, the best detection limit was the 1:100 dilution of feces in assay buffer (1.4 ng/mL), but the dilution factor shifted the limit of detection (LOD) to ~140 ng/mL. The best factored LOD of CT in feces by the VET-RPLA assay was at the 1:2 dilution, detecting approximately 19 ng/mL (Table 1).

**Quantification of CT by LPCR** was performed in triplicate as described (9). The capture antibody to CT was clone 3D11 (Biodesign). Ten-fold dilutions of CT in matrices were added (100 µL; sample volume = total assay volume). Following a 1 hr incubation and washing, detection liposomes with G\textsubscript{M1} receptors for CT on the outside, and an 84-mer qPCR amplicon on the inside, were added (8,9). DNase I digestion and lysis allowed for quantitation by qPCR cycle threshold (C\textsubscript{T}) values using Taqman reagents on a 7500 genetic analyzer (Applied Biosystems, Foster City, CA). C\textsubscript{T} values were plotted against the log of the CT concentrations and detection limits were determined as the values intersecting δ, where δ = average C\textsubscript{T} (negative controls) minus 3× the standard deviation of the negative controls. In PBS containing 1% BSA (assay buffer) or in 90% environmental water, the detection limits were < 1 pg/mL (Table 1). A fixed
concentration of CT (100 pg/mL) was examined in different dilutions of feces (1:2 through 1:100). The results were dose-dependent, with higher $C_T$ values corresponding to greater amounts of feces. When the concentration of CT was varied and tested in each fixed dilution of feces, the best detection limits were obtained in 1-20% feces in assay buffer (Table 1).

Accounting for the dilution factor from 100% feces, the 1:5 and 1:10 dilutions of feces in assay buffer were the optimal preparations, with factored LODs from 5-10 pg/mL and upper detection limits of 100 ng/mL, yielding a dynamic range of $10^4$. For clarity, a 10% stool sample containing 10 ng/mL that agglutinated down to the 3rd 1:2 serial dilution would have a detection limit of 1.25 ng/mL CT and a factored LOD of 12.5 ng/mL CT.

A rapid field assay for CT must give meaningful results on watery stool or environmental water samples. CT levels in human stool samples from patients with acute infection are ~100 ng/mL (2,11,13). The CT concentration that differentiates asymptomatic from mild *Vibrio cholera* infection ranges from 10-100 pg/mL (2). Accordingly, a clinical test for CT should cover the range of 10 pg/mL to 100 ng/mL. As ~50% of clinically relevant CT concentrations are less than 1 ng/mL (11), it is likely that the VET-RPLA, and additional assays quoted here would fail to identify CT in some 50% of samples, even under ideal conditions.

Some limitations have been suggested for the LPCR assay. First, samples with PCR inhibitors may give false negative results (11,13). LPCR avoids this by employing multiple steps that include washes following the application of sample prior to PCR. The amplicon employed in the LPCR assays was from a $\beta_2$-microglobulin transcript that spans two introns and thus is not present in biological samples (8,9). Also, PCR inhibitors are most effective upon very low amounts of target. LPCR encapsulates ~70 amplicons per liposome as a pre-amplification factor. Second, the qPCR output of the LPCR assay may be difficult to adapt to field conditions.
Portable qPCR instruments the size of a briefcase have been available for well over a decade (10). These are successfully marketed and several companies offer lyophilized reagents. LPCR avoids the need for stringent PCR laboratory conditions because DNase I digestion degrades contaminating DNA prior to qPCR (8, 9).

In conclusion, LPCR detected CT in the range of 10 pg/mL to 100 ng/mL in fecal and environmental samples. This was order(s) of magnitude more sensitive than the VET-RPLA standard. It follows that the LPCR assay is likely to positively identify clinically/field relevant concentrations of CT in the vast majority of fecal and environmental samples.

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REFERENCES


Table 1. Effect of matrix upon the lower limits of cholera toxin quantified by assay*.

<table>
<thead>
<tr>
<th>Sample matrix</th>
<th>LPCR assay</th>
<th>VET-RPLA</th>
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<tbody>
<tr>
<td></td>
<td>Detection limit (pg/mL)</td>
<td>Factored LOD a (pg/mL)</td>
</tr>
<tr>
<td></td>
<td>Linear Range b (pg/mL)</td>
<td></td>
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<tr>
<td>Buffer alone c</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Simulated feces 1:100</td>
<td>1 ± 0.2 d</td>
<td>80-120</td>
</tr>
<tr>
<td>Simulated feces 1:10</td>
<td>2 ± 0.9</td>
<td>10-30</td>
</tr>
<tr>
<td>Simulated feces 1:5</td>
<td>3 ± 2</td>
<td>5-30</td>
</tr>
<tr>
<td>Simulated feces 1:2</td>
<td>30 ± 10</td>
<td>40-80</td>
</tr>
<tr>
<td>environmental water 9:10</td>
<td>&lt; 1</td>
<td>&lt; 1.1</td>
</tr>
<tr>
<td>environmental water 1:2</td>
<td>nd</td>
<td>nd</td>
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*Data represent detection limits and factored limits of detection (LOD) for the liposome-PCR (LPCR) and the *V. cholerae* and *E. coli* heat-labile enterotoxin reversed passive latex (VET-RPLA) assays, respectively. The linear range of the LPCR assay is also expressed. a Factored LOD ranges are the (detection limit ± SD) × dilution factor. Absolute detection amounts can be calculated as detection limit × dilution factor × assay volume (100 and 50 µL for LPCR and VET-RPLA, respectively). b The subset of data with linear dose-response curves were determined from mean values and SD with OriginLab 7 software (Northampton, MA). The reported ranges had goodness of fits (R) ≥ 0.97. c Buffer alone was the assay buffer described above for LPCR and the manufacturer’s buffer for VET-RPLA. d Analyses of the detection limits of the 1:2, 1:10, and 1:100 dilutions of feces between the LPCR and VET-RPLA assays using the 2-tailed, homoscedastic Student’s t-test gave P < 0.01. e nd; no data available.