The Liposome PCR Assay Is More Sensitive than the *Vibrio cholerae* Enterotoxin and *Escherichia coli* Heat-Labile Enterotoxin Reversed Passive Latex Agglutination Test at Detecting Cholera Toxin in Feces and Water[▽]

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Practical detection of cholera toxin (CT) by a liposome PCR (LPCR) immunoassay was compared to that of an established *V. cholerae* enterotoxin and *Escherichia coli* heat-labile enterotoxin reversed passive latex agglutination (VET-RPLA) assay. LPCR detected CT in the range of 10 pg/ml to 100 ng/ml in simulated feces and environmental water. Detection by VET-RPLA required at least 4 to 19 ng/ml CT.

Diarrhea transmitted by the fecal-oral route is a major public health burden in the developing world. The enterotoxin of *Vibrio cholerae* (cholera toxin [CT]) 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 bacteria of 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 enzyme-linked immunosorbent assay (ELISA) or agglutination immunoassays. We reported highly sensitive (subattomolar) detection of CT by a liposome PCR (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 that of 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 methods with detection limits of some 600 molecules (4), the required antibodies are not commercially available (references 1, 4, 5, 11, and 13 and references therein). The VET-RPLA assay is a good standard because its detection limits (1 to 2 ng/ml) are essentially the same as can be obtained by modern ELISAs (1, 7) and its sensitivity is equal to those of PCR and classical microbiological methods (5, 6).

Matrices. Deidentified normal human feces was from BioChemed Services (Winchester, VA). To approximate “rice-

water” consistency, stool was extracted with 1% casein in phosphate-buffered saline (PBS) (1 ml buffer was added to 1 g stool), clarified, and filtered with a 0.2- μ m filter. Muddy water was collected from a local ditch, spiked with 1% (vol/vol) 1 M phosphate buffer, pH 7, and treated as described 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-h bacterial amplification. Assay volumes consisted of 25 μ l sample plus 25 μ l of the manufacturer’s buffer. The highest 2-fold dilution that produced positive agglutination in a 24-h period defined the detection limit. We reproduced the VET-RPLA detection limits ($\sim$ 1 ng/ml) of CT (Sigma; C8052) described by others (1; VET-RPLA package insert). Tenfold 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 $\sim$ 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 $\sim$ 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 previously (9). The capture antibody to CT was clone 3D11 (Biodesign). Tenfold dilutions of CT in matrices were added (100 μ l; the sample volume was the total assay volume). Following a 1-h incubation and washing, detection liposomes, with G_{M1} receptors for CT on the outside and an 84-mer quantitative-PCR (qPCR) amplicon on the inside, were added (8, 9). DNase I digestion and lysis allowed for quantitation by qPCR of cycle threshold (C_T) values using TaqMan reagents on a model 7500 genetic analyzer (Applied Biosystems, Foster City, CA). C_T values were plotted against the log of the CT concentrations, and detection limits were determined as the values intersecting δ , where δ is the average C_T (negative controls) minus 3 $\times$ the standard deviation (SD) of the C_T values for the negative controls. In PBS containing 1%

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TABLE 1. Effect of matrix upon the lower limits of cholera toxin quantified by assay^f

Sample matrix	Dilution	LPCR assay			VET-RPLA	
		Detection limit (pg/ml)	Factored LOD (pg/ml) ^a	Linear range (pg/ml) ^b	Detection limit (pg/ml)	Factored LOD (pg/ml)
Buffer alone ^c		<1	<1	1–1,000	800 ± 180	600–1,000
Simulated feces	1:100	1 ± 0.2 ^d	80–120	10–10,000	1,400 ± 650	75,000–210,000
	1:10	2 ± 0.9 ^d	10–30	10–10,000	4,100 ± 940	32,000–50,000
	1:5	3 ± 2	5–30	1–1,000	ND ^e	ND
	1:2	30 ± 10 ^d	40–80	100–100,000	9,300 ± 2,200	14,000–23,000
Environmental water	9:10	<1	<1.1	ND	ND	ND
	1:2	ND	ND	1–1,000	2,000 ± 1,800	400–7,600

^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*) of ≥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 two-tailed, homoscedastic Student *t* test gave a *P* of <0.01.

^e ND, no data available.

^f 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 agglutination (VET-RPLA) assays, respectively. The linear range of the LPCR assay is also expressed.

bovine serum albumin (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 to 20% feces in assay buffer (Table 1). After we accounted 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 of from 5 to 10 pg/ml and upper detection limits of 100 ng/ml, yielding a dynamic range of 10⁴. 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 cholerae* infection ranges from 10 to 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 assay 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 β2-microglobulin transcript that spans two introns and thus is not present in biological samples (8, 9). Also, PCR inhibitors are most effective upon very small amounts of target. LPCR encapsulates ~70 amplicons per liposome as a preamplification 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 an 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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