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Use of a real time PCR assay for detection of the *ctx*A gene of *Vibrio cholerae* in an environmental survey of Mobile Bay

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Abstract

Toxigenic *Vibrio cholerae*, the etiological agent of cholera, is a natural inhabitant of the marine environment and causes severe diarrheal disease affecting thousands of people each year in developing countries. It is the subject of extensive testing of shrimp produced and exported from these countries. We report the development of a real time PCR (qPCR) assay to detect the gene encoding cholera toxin, *ctx*A, found in toxigenic *V. cholerae* strains. This assay was tested against DNA isolated from soil samples collected from diverse locations in the US, a panel of eukaryotic DNA from various sources, and prokaryotic DNA from closely related and unrelated bacterial sources. Only *Vibrio* strains known to contain *ctx*A generated a fluorescent signal with the 5′ nuclease probe targeting the *ctx*A gene, thus confirming the specificity of the assay. In addition, the assay was quantitative in pure culture across a six-log dynamic range down to <10 CFU per reaction. To test the robustness of this assay, oysters, aquatic sediments, and seawaters from Mobile Bay, AL, were analyzed by qPCR and traditional culture methods. The assay was applied to overnight alkaline peptone water enrichments of these matrices after boiling the enrichments for 10 min. Toxigenic *V. cholerae* strains were not detected by either qPCR or conventional methods in the 16 environmental samples examined. A novel exogenous internal amplification control developed by us to prevent false negatives identified the samples that were inhibitory to the PCR. This assay, with the incorporated internal control, provides a highly specific, sensitive, and rapid detection method for the detection of toxigenic strains of *V. cholerae*.
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Keywords: Vibrio cholerae; Real time PCR; Cholerae toxin

1. Introduction

Cholera continues to be an important disease globally from the perspective of morbidity and mortality as well as from the potential economic consequences from its presence in foods traded internationally (FAO, 2003). While *Vibrio cholerae* is a common, naturally-occurring estuarine bacterium in tropical and temperate climates, strains causing the disease *cholera gravis* are relatively rare and typically of human fecal origin. The vast majority of strains associated with epidemic cholera are attributed to toxigenic *V. cholerae* with the O1 serotype (Nishibuchi and DePaola, 2005). Within the last 10 years, the polysaccharide capsule producing O139 serotype has also been associated with toxigenic cholera cases (Kaper et al., 1995; Albert et al., 1993; Islam et al., 1994; Ramamurthy et al., 1993). Recently, another serotype (O141) was demonstrated to contain the *ctx* operon (Dalsgaard et al., 2001). The filamentous bacteriophage CTXΦ confers the *ctx* operon to *V. cholerae* strains as a prophage that carries the *ctx*A and *ctx*B genes. The *ctx*AB genes encode for cholera toxin (CT), which is responsible for the life threatening diarrheal disease *cholera gravis* caused by epidemic cholera (Karunasagar et al., 1995). Some strains of *V. cholerae* that lack *ctx* can also cause human illness but these illnesses are much less severe and rarely life threatening (Anderson et al., 2004; Kaper et al., 1995). The

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presence of *ctx*AB can be used to distinguish toxigenic cholera from most non-toxigenic *V. cholerae* strains.

In developing countries, contaminated potable water is the primary route of transmission of toxigenic cholera (Swerdlow et al., 1992). Seafood leads all other foods in international trade and can become contaminated by toxigenic or non-toxigenic *V. cholerae* during production, handling, or processing. Shrimp is the leading seafood export of many of the countries where cholera is endemic. These countries often produce shrimp in aquaculture ponds that can be contaminated with human fecal waste, which may lead to contamination with toxigenic *V. cholerae* (Gopal et al., 2005; Koonse et al., 2005).

The current FDA Bacteriological Analytical Manual (BAM) recommends enrichment of 25 g of raw oysters at 42 °C for the recovery of *V. cholerae* (DePaola and Kaysner, 2001). After enrichment, samples are screened for toxigenic *V. cholerae* with a conventional PCR assay targeting a portion of the *ctx* operon. We developed a real time PCR assay (qPCR) to detect toxigenic *V. cholerae*, utilizing the same enrichment procedures as recommended in the BAM. This assay is intended for screening seafood or environmental samples and testing purified isolates for the *ctx*A gene. The *ctx*A gene was targeted since its activated product is responsible for the intracellular toxicity of the CT. The real time *ctx* assay is more specific, sensitive, faster, minimizes cross contamination by using a closed tube system, and is less resource intensive than conventional PCR (Higuchi et al., 1992) or culture methods. Additionally, a novel exogenous internal amplification control was included to prevent reporting of false negatives. The assay was applied to an environmental screening of Mobile Bay, AL for the presence of non-indigenous species including toxigenic *V. cholerae*.

2. Materials and methods

2.1. Real time PCR detection of ctx-positive V. cholerae

The qPCR cycling protocol and reaction component concentrations were optimized for detection of the *ctx*A gene. The 25 μL reaction contained the following (final concentrations): 1X PCR Buffer (Invitrogen, Carlsbad, CA); 5 mM $MgCl_2$; 0.2 mM dNTP (Roche, Indianapolis, IN); 1.25 units Platinum Taq polymerase (Invitrogen); 250 nM sense (5′-tttgttaggcacgatgatggat-3′) and anti-sense (5′-accagacaatatagtttgacccactaag-3′) primers (Integrated DNA Technologies, Coralville, IA) generating an 84 base pair amplicon within the *ctx*A gene; 100 nM 5′ nuclease probe within the *ctx*A gene (tgtttccacctcaattagtttgagaagtgccc) 5′ labeled with either FAM or TET and a Black Hole Quencher 1 on the 3′ end (Integrated DNA Technologies); amplification primers and a Cy5 labeled probe to detect the exogenous internal amplification control (IAC) (patent pending; currently licensed by Cepheid, Sunnyvale, CA); and 2–2.5 μL of template.

Real time PCR thermal cycling was run using the SmartCyclerII® system (Cepheid) utilizing an initial denaturation and polymerase activation step at 94 °C for 2 min, followed by 45 cycles of denaturation for 94 °C for 10 s, and a combined anneal and extension step at 63 °C for 30 s with the instrument optics on. Default analysis parameters were used except that the manual threshold fluorescence units setting was adjusted to 10.

2.2. Real time PCR specificity for ctx-positive V. cholerae

The *ctx*A qPCR fluorogenic probe and primer set were tested for specificity against DNA from 16 bacterial genera, 13 eukaryotic species, and 13 soil samples from various locations across the US (Table 3). Specificity testing of eukaryotic and soil DNA was provided by Lawrence Livermore National Laboratories, Livermore, CA.

2.3. Standard curves for real time PCR assay

Template for generation of standard curves was prepared by growing a mid-exponential phase culture of the *ctx*AB positive control strain *V. cholerae* 6706 (a Latin American toxigenic strain obtained from CDC) in alkaline peptone water (APW; 1% peptone, 1% NaCl, pH 8.5±0.2). Ten-fold serial dilutions were prepared in sterile phosphate buffered saline (PBS; 7.65 g NaCl, 0.724 g Na_2HPO_4 anhydrous, 0.210 g KH_2PO_4 per liter, pH 7.4). Aliquots of each dilution were spread (in triplicate) onto T_1N_1 agar (1% tryptone, 1% NaCl, 2% agar, pH 7.6±0.2) plates and colonies were counted after overnight incubation at 35 °C. A second aliquot of each dilution was boiled for 10 min and used for template in the qPCR assay.

The standard curve was generated by plotting the log value of the calculated colony forming units (CFU) per reaction versus the cycle threshold (Ct). The Ct is defined as the cycle where the fluorescent signal is sufficient to cross a defined threshold. Standard curves were generated for each particular matrix (Fig. 1). The standard curves were repeated in the presence of an internal amplification control (IAC), and in the presence of 2 μL from an overnight 1:10 APW enrichment of 25g shrimp, or a 1:100 APW enrichment of 25 g of oyster. The shrimp and oysters were purchased from a local vendor and harvested from Cedar Point, AL, respectively. The qPCR efficiency of the assay was calculated using the formula efficiency = $10^{\wedge[-1/\text{slope}]}$ (http://efficiency.gene-quantification.info/).

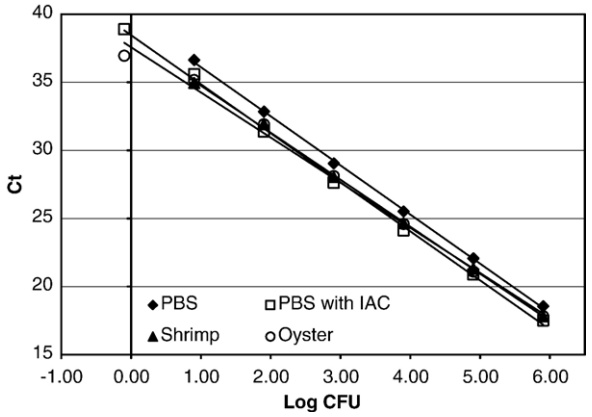

Fig. 1. Standard curves using *ctx*A qPCR showing correlation between the Log value of 10-fold serial dilutions of *V. cholerae* 6706 and respective Ct values obtained in PBS, PBS with IAC, shrimp, and oyster matrices with a detection limit of less than 10 CFU/reaction.

Table 1
Environmental samples from Mobile Bay tested by qPCR

Number	Samples		qPCR	
	Type	Source	IAC [a]	*ctx*A
A-218/219	Sediment	Gas platform	+	ND
A-249	Sediment	Gas platform	+	ND
A-245	Oyster	Oyster reef	+	ND
A-244	Oyster	Oyster reef	+	ND
A-120–125	Ballast water	Cargo ship	+	ND
A-126/127	Ballast water	Cargo ship	+	ND
A-166	Clams	Shoreline	+	ND
A-167	Sediment	Shoreline	+	ND
A-168	Clams	Shoreline	+	ND
A-169	Sediment	Shoreline	+	ND
A-170	Clams	Marina	+	ND
A-171	Sediment	Marina	+	ND
A-155	Oyster	Lighthouse	+	ND
A-74	Sediment	Shoreline	ND [b]	ND
A-133	Sediment	Shoreline	ND	ND
A-134	Sediment	Shoreline	ND	ND

[a] Internal Amplification Control.
[b] ND = not detected.

2.4. Environmental sample collection and processing

Shellfish ($n=3$ oysters; $n=3$ clam), sediments ($n=8$), and ballast water ($n=2$) (Table 1) were collected from various locations in Mobile Bay, AL from September 2–4, 2003, for analysis of non-indigenous microorganisms. Oyster samples were collected by oyster tongs or by hand; sediment samples were collected into a sterile specimen cup by removing the upper 10 mm of sediment with a sterile spatula; and ballast water samples were collected by lowering a sterile test tube (15–50 mL depending on the sampling aperture) into the ballast compartments of the ships to obtain 100 mL. All samples were placed on bagged ice and transported to the Gulf Coast Seafood Laboratory (GCSL) for analysis within 6 h of collection.

Oyster samples (10–12 animals) were scrubbed and shucked according to APHA guidelines (American Public Health Association, 1970) and homogenized in a sterile blender for 90 s. Oyster homogenate (25 g) was added to 2475 mL of pre-warmed (42 °C) APW in a sterile 4 L flask and the sample was incubated overnight at 42 °C (DePaola and Kaysner, 2001). A 1-mL aliquot of enrichment was boiled for 10 min and 2–2.5 μL of the boiled aliquot was used as template in the *ctx*A qPCR assay (Blackstone et al., 2003).

Twenty-five grams of sediment and ballast water were weighed into 225 mL of APW pre-warmed to 42 °C in a sterile 1 L flask and incubated overnight at 42 °C. A 2–2.5 μL portion of boiled template, prepared as described above, was tested for *ctx*A by qPCR.

2.5. Isolation and characterization of suspect *V. cholerae*

The pellicle growth from overnight APW enrichments was streaked to four Thiosulfate–Citrate–Bile salts–Sucrose (TCBS) agar plates and incubated at 35 °C overnight. Typical colonies (yellow; sucrose positive) were transferred into 96-well microtiter plates containing 100 μL APW per well and incubated at 35 °C with gentle rocking. After 4–6 h, a 48-prong replicator (Boekel, Feasterville, PA) was used to transfer growth to T_1N_1 plates; the plates were incubated overnight at 35 °C. Colony lifts were performed as previously described (McCarthy et al., 2000) and DNA probe hybridization for detection of *V. cholerae* species was performed using a novel AP-labeled probe (5′-Xgcaaaatctgtctcgcactcatgt-3′), where X is alkaline phosphatase (AP) conjugated 5′ amine-C6 (DNA Technologies, Denmark). Hybridization, wash, and signal development conditions were as previously described for detection of the *tdh* gene of *Vibrio parahaemolyticus* (McCarthy et al., 2000). As the AP-probe was previously determined to cross-react with some *Vibrio mimicus* and *Vibrio metschnikovii* strains (data not shown), this hybridization procedure was used as a screening method only and cultures that were positive with the AP probe were isolated and subjected to further analysis.

AP-probe-positive isolates were grown in 1% tryptone, without added NaCl and streaked onto T_1N_1 agar and tested by slide agglutination using *V. cholerae* O1 and O139 antisera as previously described (DePaola and Kaysner, 2001). Agglutination was compared to the sterile saline negative control and the *V. cholerae* VRL-1984 and F-832 positive controls (Table 2) for the detection of the O1 and O139 serotypes, respectively. Representative isolates were confirmed as *V. cholerae* by API 20E.

Table 2
Vibrio cholerae strains tested by qPCR for presence of *ctx*A

V. cholerae strain	Serotype	Isolate notes	*ctx* qPCR
UCIC 305 E	Non-O1	CA, water	−
UNB 420 I	Non-O1	CA, water	−
UNB 420 L	Non-O1	CA, water	−
10–49	Non-O1	FL, oyster	−
17–17	Non-O1	FL, oyster	−
133–29	Non-O1	FL, oyster	−
167–19	Non-O1	FL, oyster	−
201–35	Non-O1	FL, oyster	−
106–48	Non-O1	FL, oyster	−
106–60	Non-O1	FL, oyster	−
106–61	Non-O1	FL, oyster	−
109–73	Non-O1	FL, oyster	−
133–67	Non-O1	FL, oyster	−
O138	Non-O1	FL, oyster	−
CDC-3523-03	O141	Hemolysin negative	−
CDC-3523-03	O141	Hemolysin positive	−
JBK 70	O1	569B *ctx* deletion mutant	−
569B [a]	O1	Classical strain	+
VRL-1984 [a]	O1	Gulf Coast strain	+
14103 [a]	O1	ATTC strain	+
6706 [a]	O1	Latin American strain	+
6707 [a]	O1	Latin American strain	+
154 [a]	O1	Classical	+
2164-78 [a]	O1	Louisiana patient, 1978	+
709-1 [a]	O1		+
1463-88 [a]	O1		+
1800-62 [a]	O1		+
246-0058 [a]	O1		+
Truk [a]	O1		+
F832 [a]	O139		+
CDC-3541.97 [a]	O141		+
CDC-3541-98 [a]	O141		+

[a] *ctx* positive strain of *V. cholerae*.

Representative suspect *V. cholerae* (one from each sample) were also grown in APW overnight at 35 °C with shaking. A 100 μL aliquot was removed and boiled for 10 min. A 2 μL aliquot of the crude lysate was tested for the presence of *ctx*A by qPCR.

3. Results

3.1. Real time PCR detection of ctx positive V. cholerae

The qPCR assay only reported a positive signal for *V. cholerae* strains known to possess the *ctx*A gene (Table 2). None of the eukaryotic DNAs (0/14), soil DNAs (0/13), and bacterial isolates (0/63), representing 16 bacterial species that lacked the *ctx*A gene, produced a fluorescent signal, as expected (Table 3). The limit of detection of the assay was less than 10 CFU per reaction for all matrices and as low as 0.8 CFU/reaction in oyster, and had a dynamic range of at least six logs (Fig. 1). The calculated efficiency of the *ctx* assay based on efficiency = $10^{(1/1-\text{slope})}$ was approximately 90% with and without the presence of the IAC. Also, the efficiency of the reaction in oyster and shrimp enrichments was sufficient to reach the <10 CFU per reaction limit of detection as found in pure culture analysis. Traditional methods for the detection of *V. cholerae* can take from 4 to 7 days to complete (BAM), while the qPCR assay used with a short enrichment (4–6 h) can be completed within 1 day with detection taking less than 1 h.

Table 3
Non-*Vibrio cholerae* samples tested by qPCR for presence of *ctx*A

DNA template	*ctx* qPCR
Bovine	–
Canine	–
Chicken	–
Drosophila	–
Feline	–
Flea	–
Mosquito	–
Murine	–
Porcine (2)	–
Rabbit	–
Rat	–
Human	–
Tick	–
Soil (13)	–
Bacillus cereus	–
B. globigii	–
B. subtilis	–
Borellia burgdorferi	–
Citrobacter freundii	–
Edwardsiella tarda	–
Enterobacter aerogenes	–
E. cloacae	–
Escherichia coli (13)	–
Haemophilus influenza	–
Morganella morganii	–
Proteus mirabilis	–
Providencia stuartii	–
Pseudomonas aeruginosa (2)	–
Salmonella typhimurium (2)	–
Serratia liquefaciens	–
Staphloccocus aureus	–
V. fluvialis (2)	–
V. furnissii	–
V. metschnikovii (6)	–
V. mimicus (12)	–
V. parahaemolyticus (2)	–
V. vulnificus (4)	–
V. mimicus WMS 33[a]	+

( )—number of samples/species tested.
[a] *V. mimicus* WMS 33 is reported to possess the *ctx*A gene.

Table 4
Comparison of qPCR and traditional analytical methods on pure cultures isolated from enriched samples described in Table 1

Sample	Traditional analyses				
	*ctx*A qPRC	Vc species Ap-probe	O1 serology	O139 serology	Growth in 0% NaCl
A-244	–	+	–	–	+
A-166	–	+	–	–	+
A-167	–	+	–	–	+
A-168	–	+	–	–	+
A-169	–	+	–	–	+
A-170	–	+	–	–	+
A-171	–	+	–	–	+
A-74	–	+	–	–	+
A-133	–	+	–	–	+
A-134	–	+	–	–	+

Only samples with an API confirmed *V. cholerae* isolate are listed.

3.2. Environmental samples

The survey of Mobile Bay, AL revealed no toxigenic *V. cholerae*. The IAC developed at GCSL (Vickery et al., 2004) indicated that three sediment samples of the 16 environmental samples (6 shellfish, 8 sediments, and 2 ballast waters) were inhibitory to the qPCR (Table 1). Inhibition of qPCR was further demonstrated by spiking positive control template into the inhibitory samples; no qPCR amplification was observed. Purification and clean-up techniques were unsuccessful at removing the inhibitors (data not shown).

AP-probe hybridization revealed 64 suspect *V. cholerae* isolates from 11 samples. Of the 64 isolates, 60 grew in 1% tryptone without added NaCl and produced yellow colonies on TCBS; these 60 isolates were considered to be *V. cholerae*. One *V. cholerae* isolated from each of the 11 samples were confirmed by API as *V. cholerae*. None of the 11 representative isolates agglutinated with the O1 or O139 antisera, nor were they *ctx*A positive by qPCR (Table 4).

4. Discussion

We developed a qPCR method for the rapid screening for toxigenic *V. cholerae* in APW enrichments of oyster, shrimp, and environmental samples. This assay is specific for the detection of *Vibrio* strains that possess the *ctx*A gene, has a sensitivity of less than 10 CFU per reaction, and a dynamic range of at least six logs. With the speed of qPCR and a 4 to 6 h enrichment, a same-day detection of toxigenic *V. cholerae* from seafood and environmental samples is possible. A similar

approach for screening seafood enrichments using conventional PCR is described in the BAM. Our qPCR assay provides a number of potential advantages over the current BAM PCR method: the use of a probe provides an additional level of specificity in qPCR assays, elimination of the gel electrophoresis step with a closed tube system saves considerable time and greatly reduces the likelihood of cross contamination with PCR amplicons, and the incorporation of an IAC identifies matrix inhibition of PCR and reduces the likelihood of false negative results.

Since the 1970s, there have been sporadic cases and a few outbreaks of cholera in the U.S. that were caused by toxigenic *V. cholerae* O1 designated as the "Gulf Coast strain" (Wachsmuth et al., 1991; DePaola et al., 1992). It is only associated with seafood from the Gulf of Mexico and is clearly distinguishable from other toxigenic *V. cholerae* including the 7th pandemic strains. In 1994, Motes et al., reported finding the "Gulf Coast strain" along the US Gulf Coast; however, this strain was not recovered in the 2003 survey. Recently, a toxigenic *V. cholerae* O141 strain emerged in the US and in Asia and caused sporadic cases associated with seafood consumption (Dalsgaard et al., 2001). The *ctx* operon of this organism appears to be the same as in other toxigenic strains and was also detected by this assay. The presence of the "Gulf Coast strain", O141, or other toxigenic non-O1 or non-O139 strains have not been associated with epidemics, and have considerably less public health significance than other toxigenic *V. cholerae*, such as the 7th pandemic O1 strains and O139 strains that are typically associated with pandemics. As such, it is strongly recommended that a positive qPCR assay for *ctx*A be followed by attempts to isolate the strain causing the positive signal to identify its serotype, and further characterization to determine the strain's epidemic potential. Seafood imported into the US is screened for toxigenic *V. cholerae*; the use of qPCR in tandem with traditional techniques could allow an increase in the sampling frequency and provide a more rapid means for detection of toxigenic *V. cholerae* in samples.

Food and environmental matrices often have materials that are inhibitory to PCR and qPCR, and could result in false negative reporting on a sample (DePaola and Huang, 1995; Gubala, 2006; Lyon, 2001). The internal amplification control in the *ctx*A assay identifies samples that are inhibitory to PCR thus precluding false negative reporting on a sample which might have contained toxigenic *V. cholerae*. The IAC developed at GCSL showed the presence of inhibitors in three of 16 samples analyzed. It is our belief that the qPCR assay developed for *ctx*A positive *V. cholerae*, when used in tandem with our IAC, provides a greater degree of confidence, speed, specificity, and sensitivity for the detection of toxigenic strains of *V. cholerae* in pure culture and environmental samples when compared to traditional culture methods.

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