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Rapid One-Step Quantitative Reverse Transcriptase PCR Assay with Competitive Internal Positive Control for Detection of Enteroviruses in Environmental Samples

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Human enteroviruses can serve as a more accurate indicator of human fecal contamination than conventional bacteriological fecal indicators. We describe here a quantitative reverse transcriptase PCR (qRT-PCR) assay specifically tailored to detect these viruses in environmental waters. The assay included a competitive internal positive control (CIPC) that allowed the inhibition of qRT-PCRs to be quantitatively assessed. Coamplification of the CIPC with enteroviral genetic material did not affect the sensitivity, specificity, or reproducibility of the enteroviral qRT-PCR assay. The assay is rapid (less than 5 h from sample to result), has a wide dynamic range (>3 logs), and is capable of detecting as few as 25 enteroviral genomes with an average amplification efficiency of 0.91. In samples with low or moderate inhibition, the delay in CIPC amplification was used to adjust enterovirus qRT-PCR concentrations to account for losses due to inhibition. Samples exhibiting significant inhibition were not corrected but instead diluted twofold and immediately assayed again. Using significantly inhibited samples, it was found that dilution relieved inhibition in 93% (25 of 27) of the samples. In addition, 15% (4 of 27) of these previously negative samples contained enteroviral genomes. The high-throughput format of the assay compared to conventional culture-based methods offers a fast, reliable, and specific method for detecting enteroviruses in environmental water samples. The ability of the assay to identify false negatives and provide improved quantitative assessments of enterovirus concentrations will facilitate the tracking of human fecal contamination and the assessment of potential public health risk due to enteroviruses in recreational and shellfish harvesting waters.

Of the enteric viruses, human enteroviruses are one of the most commonly detected viruses in polluted waters (25). Enteroviruses are members of the family *Picornaviradae* and are estimated to cause 30 million to 50 million infections per year in the United States, with 30,000 to 50,000 of these resulting in meningitis hospitalizations (22). Due to viral replication in the gastrointestinal tract, an infected individual may shed enteroviruses from the stool for up to 16 weeks (24), with densities as high as 10^6 viruses per g of feces (16). Enteroviruses are tolerant to residual chlorine from sewage treatment (12) and a wide range of temperatures and salinities (27, 29), facilitating their survival in environmental waters. Routine monitoring of indicator bacteria in environmental waters has demonstrated weak or nonexistent predictive relationships to enteroviruses and other human pathogens, suggesting a need for viral pathogen-specific assays in environmental waters (8, 9, 19). Given the load of enteroviruses shed into waters impacted by sewage, their persistence in environmental waters compared to indicator bacteria, and the role of enteroviruses in waterborne disease, there is clearly a need for sensitive, quantitative assays for enteroviruses in environmental samples.

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Quantitative reverse transcriptase PCR (qRT-PCR) offers the potential for fast, reliable, high-throughput analysis of water samples for enteroviruses, traits not found with traditional cell-culture-based detection approaches. Indeed, TaqMan-based qRT-PCR assays have been developed for the detection and quantification of viral pathogens from a variety of sources, including norovirus genogroups I and II from stool and shellfish (11); hepatitis A virus (10); and enteroviral loads in cerebrospinal fluid (17), sludge (18), and natural water samples (4, 6, 9, 23). Despite the fact that these and other successful PCR-based assays for the detection of enteroviruses have been developed (see, for example, references 18, 19, 24, 26, and 28), major hurdles remain for the accurate quantification of viruses from environmental waters using PCR-based approaches.

One of the primary obstacles to successful PCR-based analysis is copurification of inhibitory compounds, such as polysaccharides and humic, fulvic, or tannic acids during RNA isolation from environmental water samples. These compounds readily inhibit qRT-PCR. Adjuvants such as T34 gene protein, polyvinylpyrrolidone (PVP), and bovine serum albumin have been used in the past to reduce PCR inhibition by these compounds (see, for example, references 4, 15, and 18). However, none have proven fully effective for qRT-PCR. Furthermore, most of these assays have not included a control that allows simultaneous assessment of PCR inhibition and enteroviral concentration.

We present here a rapid, sensitive qRT-PCR assay for quantifying enteroviral concentrations in natural waters that in-

TABLE 1. Primer and probe sequences

Primer or probe	Sequence
EV6F (forward)	5′-GGTGGTCCAGGCTGCGTTGG-3′
EV8R (reverse)	5′-CCCATGTCCCGCAGTGCATC-3′
EV1F T7 comp (forward)	5′-CACG***TAATACGACTCACTATAGG***GCCC**TGAATGCGGCTAAT***CTTCGCTATTACG CCAG*-3′*a*
EV1R comp (reverse)	5′-GT**TGTCACCATAAGCAGCCA***TTACAAC GTCGTGACTG*-3′*a*
EV1F (forward)	5′-CCCTGAATGCGGCTAAT-3′*b*
EV1R (reverse)	5′-TGTCACCATAAGCAGCCA-3′*b*
EV probe	5′-[FAM]-ACGGACACCCAAAGTAGTCG GTTC-[BHQ-1]-3′*b*
CIPC probe	5′-[Cy5]-TGTGCTGCAAGGCGATTAAGT TGGGT-[BHQ-2]-3′*a*

a Sequences modified or taken directly from Kleiboeker (13); portions homologous to the plasmid vector are in italics, sequences homologous to enteroviral primer sequences (EV1F and EV1R respectively) are shown in boldface, and the T7 RNA polymerase promoter is shown in bold italics.

b Sequences provided by G. Shay Fout (U.S. Environmental Protecction Agency, NERL, Cincinnati, Ohio).

cludes a competitive internal positive control (CIPC) designed to quantify qRT-PCR inhibition. This control consisted of spiking each sample with a synthetic target (CIPC), which was reverse transcribed and amplified by using the same primers used in the enteroviral assay. During amplification, the CIPC was distinguished from enteroviral genomes by using two different fluorescently labeled TaqMan probes specific for each of the two templates. Adding sufficiently low concentrations of the CIPC to each sample prevented significant competition for the primer or nucleotide pools. This allowed unencumbered detection of enteroviruses over a wide range of concentrations. Simultaneously, the degree of PCR inhibition was estimated by the delay in the sample extract CIPC cycle threshold values relative to uninhibited control reactions. This permitted rapid identification of samples which required reanalysis after dilution to reduce inhibition. The CIPC further allowed consistent normalization of results from one experiment to the next and, over a certain range of inhibition, corrected estimates of the number of viral genomes that would have been detected if no inhibition had occurred.

MATERIALS AND METHODS

Development of the qRT-PCR assay. A TaqMan-based qRT-PCR assay was developed to detect enteroviral genomes. The assay is based on pan-enteroviral primers and a TaqMan probe, specific for a 143-nucleotide portion of the 5′ untranslated region (5′UTR) of poliovirus. This 5′UTR region is highly conserved in the 62 nonpolio enteroviruses and three poliovirus types examined to date. It is also the target region upon which several other enteroviral RT-PCR assays have been based (see, for example, references 18, 19, and 24). The enterovirus (EV)-specific TaqMan probe (Table 1) was labeled with the 5′ reporter fluorophore FAM (6-carboxyfluorescien) and the 3′ quencher dye BHQ-1 (black hole quencher 1). After demonstrating no significant difference in the efficiency of optimized one-step and two-step qRT-PCRs (5), we chose a one-step, single-tube format using the QIAGEN (Valencia, California) One-Step RT-PCR kit. Thermal cycling and fluorescence detection were carried out by using a Cepheid Smart Cycler II (Sunnyvale, Calif.). Reaction conditions consisted of 1× RT-PCR buffer, 6 mM $MgCl_2$, 500 μM deoxynucleoside triphosphates (dNTPs), 700 nM primers EV1 F and EV1R (Table 1), 120 nM concentrations each of the EV and the competitive internal positive control (CIPC) probes (Table 1, see CIPC information below), 30 μg of bovine serum albumin (Calbiochem, Bloomington, Ind.), 20 U of recombinant RNasin (Promega, Madison, Wis.), 1.5% PVP-25 (Sigma Chemical Co., St. Louis, Mo.), 100 copies of the CIPC template, enteroviral standard or 5 μl of extracted RNA from a field sample, and 1 μl of enzyme mix in a final reaction volume of 25 μl. A standard curve was created from serially diluted enteroviral standards and, respectively, assaying 25, 250, 2,500, or 25,000 copies per reaction. The reverse transcription was performed for 1 h at 50°C, followed by a 15-min incubation at 95°C for *Taq* activation and inactivation of the RT enzymes. Enteroviral cDNA was amplified by 45 cycles of denaturation (94°C for 15 s) and annealing/extension (60°C for 1 min, with SmartCycler II optics on for fluorescent signal detection).

Real-time fluorescence measurements were recorded on the SmartCycler II instrument, with the fluorescent thresholds set manually to 5. Cycle thresholds (C_T), the cycle at which sample fluorescence exceeds background fluorescence, were recorded for the enteroviral standards, CIPC, and RNA from extracted field samples. The numbers of enteroviral genomes were interpolated from the standard curve generated from the enteroviral quantification standards versus their C_T. In reactions where the enteroviral C_T is lower than the lowest point on the enteroviral standard curve (25 copies), the concentration of genomes per reaction is interpolated from a linear regression of the enteroviral standard curve.

Development of enteroviral quantification standard for the qRT-PCR assay. A synthetic enteroviral quantification standard for quantifying the number of enteroviral genomes in the qRT-PCR assay was constructed by cloning nucleotides 348 to 1218 from the 5′ portion of poliovirus Sabin type 1 genome (GenBank Accession number AY184219) into the pCRII-TOPO T7 vector (Invitrogen, Carlsbad, Calif.). This 871-bp region encompasses the 143-bp enteroviral qRT-PCR 5′UTR target. Constructing a standard which extended beyond the primer and probe binding sites was done so that the secondary structure of the standard RNA transcripts would more closely mimic that found in a wild-type enteroviral genome than if the enteroviral quantification standard transcript were the same size as the assay target sequence. The first step in creating the enteroviral quantification standard involved a two-step qRT-PCR, performed with extracted poliovirus Sabin type 1 RNA acting as the template for the reverse transcription and subsequent PCR amplification. OmniScript (QIAGEN) reverse transcription reactions consisting of 1× RT-Buffer, 500 μM dNTPs, 1 μM EV8R (Table 1), 5 U of recombinant RNasin (Promega), and 1 μl of OmniScript RT were held at 37°C for 1 h, followed by a 15-min hold at 95°C. Double-stranded cDNA was created using 3 μl of the RT reaction using 1× PCR buffer, 2.5 mM $MgCl_2$, 500 μM dNTPs, 500 nM primers EV6F and EV8R (Table 1), and 1.25 U of Ex *Taq* R DNA polymerase (Takara, Madison, Wis.). Thermal cycling was performed on a Techne Genius thermal cycler (Burlington, N.J.), with an initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 56°C for 45 s, and 72°C for 3 min, followed in turn by a final extension at 72°C for 7 min. Primers EV6F and EV8R (Table 1) synthesized by MWG Biotech (High Point, N.C.) were designed in house to amplify an 871-nucleotide poliovirus amplicon. The PCR product was visualized through 10 mm of acrylic shielding, under low UV transillumination on a GelDoc 2000 imager (Bio-Rad, Hercules, California) in a 1.2% agarose–1× TAE gel containing 500 ng of ethidium bromide per ml. The 871-bp band was excised, gel purified using Wizard SV gel and PCR clean up system (Promega), TOPO TA cloned into a pCR II-TOPO T7 vector (Invitrogen), and transformed into One-Shot TOP10 (Invitrogen) chemically competent *Escherichia coli* according to the manufacturer's instructions. Transformed colonies were screened for correct insertion and orientation of the enteroviral product, through a PCR utilizing primer M13 forward and the internal enteroviral primer EV1R (Table 1). Plasmid DNA was isolated from positive clones using a PerfectPrep Plasmid Minikit (Eppendorf, Westbury, Conn.) and sequenced bidirectionally to confirm that the sequence and insertion of the 871-bp enteroviral 5′UTR construct was correct.

Enteroviral quantification standards were created by in vitro transcription of BamHI (New England Biolabs, Ipswich, Mass.)-linearized plasmid DNA by using a MAXIscript In Vitro T7 Transcription Kit (Ambion, Austin, Tex.). Transcripts were treated with DNase I (Ambion) and purified by using a MEGAclear RNA purification kit (Ambion). Transcript integrity was confirmed by electrophoresis for 15 min at 100 V in a 0.65% agarose–1× TAE (Tris-Acetate EDTA) gel containing 500 ng of ethidium bromide per ml and visualized under UV transillumination. The transcripts appeared as a concise band of the appropriate size on the gel, indicating that no significant degradation had occurred. Transcripts were quantified fluorometrically by using RiboGreen (Molecular Probes, Eugene, Oreg.). The enteroviral quantification standard was diluted to 10^5 copies μl^{-1} and stored in single-use aliquots at −80°C. Given that enteroviruses carry one 5′UTR target per genome (24), it is assumed that one enteroviral quantification standard transcript is equivalent to a single enteroviral genome.

Development of the CIPC. For our enteroviral qRT-PCR, a CIPC was created to assess inhibition, using the approach described by Kleiboeker (13). In brief, hybrid primers were constructed and designated EV1F T7 comp and EV1R comp. EV1F T7 comp contained, in 5′-to-3′ order, the sequence for the T7 RNA

polymerase promoter site, the enteroviral forward primer EV1F, and 17 bp homologous to bp 477 to 493 in the pCRII-TOPO T7 vector. EV1R comp contained from 5′ to 3′ the reverse enteroviral primer EV1R and 17 bp homologous to bp 403 to 419 in the vector (Table 1). These hybrid primers were used to amplify a 91-bp fragment of the TOPO vector flanked by the combined T7-EV1F sequence at the 5′ end and the EV1R sequence at the 3′ end. The amplification conditions were identical to those outlined by Kleiboeker (13). The resulting PCR product was run out on a 2% agarose–1× TAE gel containing 500 ng of ethidium bromide per ml. The expected 152-bp product was excised from the gel under low UV illumination on a GelDoc 2000 imager (Bio-Rad) and purified by using a Wizard SV gel and PCR clean-up system (Promega). Purified CIPC template DNA was transcribed at 37°C for 1 h, using a MAXIscript In Vitro T7 Transcription Kit (Ambion), yielding a 131-nucleotide transcript. The resulting RNA was purified (MEGAclear RNA Purification Kit; Ambion) and quantified fluorometrically as described for the enteroviral standard. Transcript integrity was confirmed by electrophoresis of the CIPC for 15 min at 100 V in a 2% agarose–1× TAE (Tris-acetate EDTA) gel containing 500 ng of ethidium bromide per ml and visualized under UV transillumination. The CIPC transcripts formed a concise band of appropriate size on the gel, indicating that no significant degradation had occurred. CIPC transcripts were stored in single-use aliquots at −80°C at concentrations of 10^4 copies μl^{-1}.

Incorporation of the enteroviral primers sites allowed amplification of both the CIPC and the enteroviral templates using the same enteroviral primers, EV1F and EV1R. The CIPC was discriminated from enterovirus by using the CIPC TaqMan probe, which corresponded to bp 436 to 461 of the pCRII-TOPO T7 vector. The probe was labeled with the 5′ reporter fluorophore Cy5 and the 3′ quencher dye BHQ-2 (black hole quencher 2). Further, the 126-bp CIPC and 143-bp enteroviral qRT-PCR products can be distinguished from each other by using polyacrylamide gel electrophoresis, ethidium bromide staining, and UV transillumination.

The CIPC was sequenced to ensure the integrity of the T7 RNA polymerase promoter site, the primer binding sites, and the internal vector sequence. This was accomplished by performing the reverse enteroviral qRT-PCR assay using CIPC transcripts with an additional 10-min 72°C final extension step. The resulting qRT-PCR product was purified by using a Wizard SV gel and PCR clean-up system (Promega), sequenced bidirectionally by MWG Biotech, Inc., and revealed the expected sequence. A BLAST search using this sequence revealed no homologues with naturally occurring sequences in GenBank (1, 2). Analysis of the CIPC and enteroviral amplicons using the JaMBW Chapter 3.1.9 Oligo Calculator (3) revealed that the GC contents of the CIPC amplicon (53%) and enteroviral amplicons (54%) were nearly identical.

The difference between the average C_T in the control samples and in the field samples was used to estimate the degree of PCR inhibition and for correcting the enteroviral estimates. Specifically, the ΔC_T value was calculated by subtracting the mean CIPC C_T value for the uninhibited control reactions from the sample CIPC C_T. The ΔC_T value is applied in the equation $(E + 1)^{\Delta \mathrm{CIPC}\ C_T}$, where E is the amplification efficiency calculated from the enteroviral standard curve using the equation: $E = [10^{(-1/\mathrm{slope})}] - 1$. This calculation provides an estimate for each qRT-PCR of the constant by which the corresponding enteroviral estimate must be multiplied in order to reflect the actual number of enteroviral copies that would have been obtained if no inhibition had occurred. This CIPC approach is similar to that described by Haugland et al. (7).

Samples in which the CIPC failed to amplify or amplified later than 3.7 cycles (equal to an ~1-log decrease in the qRT-PCR amplification relative to the control) greater than the average C_T of 34.6 value were considered either too inhibited for reliable quantification of enteroviral concentrations or potentially negative. To distinguish between these two possibilities, these samples were diluted to half-strength with molecular-grade RNase-free water and reanalyzed. Diluted samples with a CIPC delay of <3.7 cycles more than the control and that still showed no enteroviral amplification were scored as having an enteroviral concentration below the limit of detection (i.e., negative). Diluted samples with a CIPC delay of <3.7 cycles and for which an enteroviral concentration could then be estimated were corrected as described above using the CIPC ΔC_T method with the inclusion of 1:2 dilution factor.

Optimizing the assay. The optimal concentration of CIPC to incorporate into the qRT-PCR assay was determined empirically by adding either 0, 10, 100, or 1,000 copies of the CIPC to reactions containing serially diluted enteroviral quantification standards consisting of 25, 250, 2,500, and 25,000 copies. The amplification efficiency and sensitivity of these standard curves was compared. The goal was to determine a CIPC concentration that amplified consistently but that did not affect the efficiency or sensitivity of enteroviral amplification over a broad range of enteroviral concentrations. Once the optimized concentration of the CIPC standard was determined, the consistency between enteroviral standard curves incorporating the CIPC control was evaluated. This was done by determining the slope and efficiency statistics from nine individual standard curves and calculating the mean and standard deviation for both parameters. The relationship between assay variability and enteroviral concentration was evaluated by plotting the standard deviation in C_T values at each enteroviral quantification standard concentration from the same nine standard curves.

Evaluating the effectiveness of the CIPC correction factor using spiked samples. We examined the effectiveness of the CIPC at identifying qRT-PCR inhibition, as well as acting as a calibrator for enteroviral concentration. Ten-liter grab samples were collected from the Rachel Carson Estuarine Research Reserve in Beaufort, N.C., and transported back to the laboratory on ice in the dark on 10 May 2005. Of these samples, the one from Deep Creek water exhibited the highest degree of turbidity (16 nephelometric turbidity units). The sample was highly colored, indicating the sample's complexity and presumably high levels of humic acids, sediment, and other environmental qRT-PCR inhibitors. This high-turbidity sample was selected for testing the inhibition assay because is was likely to represent a worst-case scenario for the carryover of compounds inhibitory to the qRT-PCR after filtration and extraction.

For the study, 100-, 250-, and 400-ml aliquots were filtered in duplicate though 0.45-μm-pore-size nitrocellulose type HA filters (Millipore). At 400 ml, the filter became clogged, and no more sample could be passed. All filters appeared dark brown after filtration. RNA was extracted and eluted with 50 μl of RNase-free water as described by Noble et al. (21). The standard enteroviral qRT-PCR was performed on each of the extracts described above. These assays showed that the native enteroviral concentrations in the samples were below detection limits. The RNA extracts were again assayed, allowing further assessment of the CIPC's effectiveness as a calibrator. For each sample, 5 μl of RNA extract was added to 20 μl of qRT-PCR master mix prepared with 1,000 copies of the enteroviral quantification standard and 100 copies of the CIPC added. Each RNA extract was then assayed by using the standard qRT-PCR assay conditions, along with equivalent control reactions containing no sample RNA or a negative extraction control. A conventional enteroviral standard curve was also run. The control reactions (no sample RNA and the negative extraction control) were used to determine the average CIPC C_T of uninhibited reactions. The ΔCIPC C_T of each sample relative to the control reactions was calculated and used to estimate the true number of viral genomes present (n) in each reaction as follows: enteroviral standard curve $\times (E + 1)^{\Delta \mathrm{CIPC}\ C_T} = n$.

Use of the CIPC correction factor to estimate enteroviral concentrations in samples exhibiting significant inhibition. The ability of the CIPC to identify inhibited samples and allow detection of enteroviral genomes in diluted aliquots of these samples was evaluated using environmental water samples from Ballona Creek, Calif. These field samples were collected and filtered as described by Fuhrman et al. (4). RNA was extracted from the field samples as described in Noble et al. (21). A total of 59 samples were available for processing during this study. Samples were analyzed by our qRT-PCR assay and were considered to be significantly inhibited if the ΔCIPC C_T of each sample for a sample was ≥3.7. A reduction of 3.7 cycles corresponds to an ~1-log reduction in amplification based on the cumulative enteroviral qRT-PCR efficiency of 0.92. Samples that exhibited no amplification of the CIPC, within the 45 cycles of the qRT-PCR, which corresponds to 11 cycles beyond the average CIPC C_T, were considered completely inhibited. Based on these criteria, 36 of the original 59 samples were inhibited. To further investigate a strategy for dealing with inhibited samples, 27 of the inhibited samples were diluted 1:2 with molecular-grade RNase-free water and reanalyzed by using the standard qRT-PCR assay.

Estimating the minimum detection limits in PFU for the echovirus 12 stock. To address the qRT-PCR assay sensitivity from a public health standpoint, the relationship between enteroviral genomes (determined by the qRT-PCR) and enteroviral particles (determined by epifluorescence microscopy) was related to infectious units in the following manner. A stock of a model enterovirus, echovirus 12, was obtained from the laboratory of Mark Sobsey at the University of North Carolina at Chapel Hill. The stock was propagated and plaque assayed using FRhK-4 cells and had a titer of 1.23×10^9 PFU ml^{-1}. The concentration of viral particles in an aliquot of the titered stock was determined by using epifluorescence microscopy as described in Noble and Fuhrman (20). Briefly, echovirus 12 was diluted, fixed with 0.02-μm-pore-size-filtered 5% formalin (final concentration), and filtered onto 0.02-μm-pore-size Anodisc 25 membrane filters (Whatman, Florham Park, N.J.). Filters were stained by using SYBR Green I nucleic acid stain (Molecular Probes) and mounted on glass slides. Slides were observed on a Nikon Eclipse E800 (Nikon, Inc., Melville, N.Y.) microscope, at ×1,000 magnification, using blue excitation, with 10 random fields of virus particles enumerated, yielding a calculation of viral particles that could be compared to the original titer (in PFU) of the echovirus stock. Subsequently, the number of genome equivalents per particle was estimated by carefully diluting

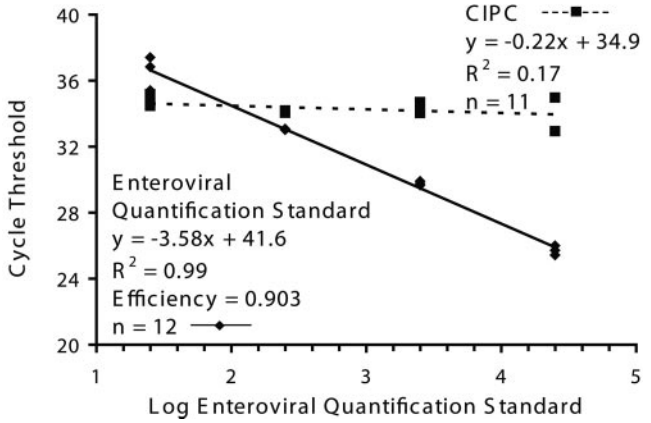

FIG. 1. Detection of the competitive internal positive control (100 copies), along with various concentrations (25 to 25,000 copies) of enteroviral quantification standards run in triplicate, to generate the standard curve used in our analysis of field samples. Enteroviral quantification standard transcripts are denoted with a "◆" symbol, while CIPC transcripts are denoted with a "■" symbol. CIPC C_T remains constant across a broad range of enteroviral standards. One of the CIPC samples failed to amplify in the presence of 25,000 copies of enteroviral standards. The amplification efficiency was calculated from the slope of the linear regression.

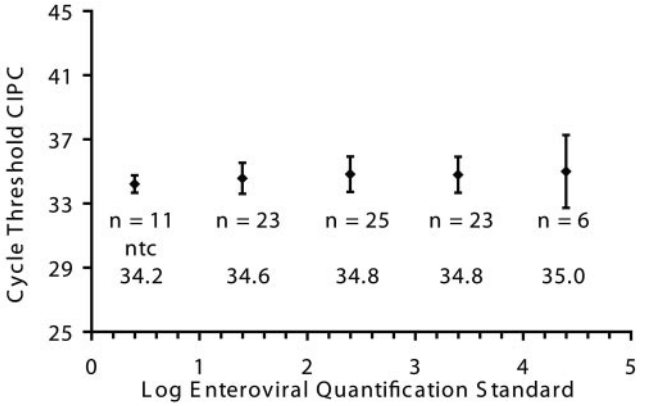

FIG. 2. Variation in CIPC C_T with various concentrations of enteroviral quantification standards ranging from 0 copies in the no-template control (ntc) to 25,000 copies. The average CIPC C_T is noted in boldface, with error bars representing ±1 standard deviation. At the highest enteroviral quantification standard concentration (25,000 copies), the CIPC failed to amplify in one-third of the samples (three of nine).

the echovirus stock so that there were either 10^9, 10^8, or 10^7 particles per 700-μl aliquot of freshly prepared RLT RNA extraction buffer (QIAGEN). At the same time, a standard curve was constructed by adding either 10^9, 10^8, or 10^7 enteroviral quantification standard transcripts to identical 700-μl aliquots of RLT RNA extraction buffer. Triplicate samples of both the viral particles and the enteroviral quantification standard transcripts from each dilution were extracted by using a QIAGEN RNeasy minikit according to the manufacturer's instructions. Final elutions to 50 μl were made by using RNase-free molecular-grade water. Then, 2 μl of the extracted RNA from each sample was assayed by using the enteroviral qRT-PCR. By extracting both the echovirus particles and the enteroviral standard transcripts in the same manner, it was possible to normalize for any losses taking place during the extraction process, thus assuring that echoviral particles and enteroviral quantification standards were subject to the same conditions prior to qRT-PCR analysis.

These data provided an estimate of both the number of viral particles PFU^{-1} and number of enteroviral quantification standard transcripts PFU^{-1}. These two estimates were used to calculate potential limits of detection in PFU for echovirus 12 as follows. For the viral particle counts it was assumed that each particle carried a single RNA genome and that each genome contained one enteroviral target sequence corresponding to one enteroviral quantification standard transcript. Given these assumptions, a lower limit of detection could be determined by dividing the minimum detectable level of enteroviral quantification standard, determined empirically for the assay, by the number of viral particles PFU^{-1}. This estimate likely represented a "worst-case" estimate for the limits of detection because evidence suggests that the SYBR Green I staining method used may underestimate the true number of viral particles present in a sample, especially when enumerating RNA viruses. Alternatively, the same calculation was done using the direct qRT-PCR estimates of the number of enteroviral genomes PFU^{-1}, which are again equivalent to enteroviral quantification standard transcripts, instead of the number of viral particles PFU^{-1}. This calculation likely represented a "best-case" estimate of the sensitivity due to the fact that it would measure any unpackaged viral genomes released during infection, in addition to intact and potentially infectious virus.

RESULTS

The reaction conditions for coamplification of the enteroviral quantification standard and the CIPC were determined after optimization of the reaction conditions as monoplex assays. Primers, probes, and nucleotide concentrations were varied factorially along with CIPC concentrations ranging from 10 to 1,000 transcripts per reaction. Through this analysis, we determined that 100 copies of the CIPC template per reaction permitted consistent amplification over a broad range of target enteroviral genomes without decreasing the sensitivity or reproducibility of the qRT-PCR (Fig. 1 and 2). As enteroviral genomes concentrations approach 25,000, however, the CIPC failed to amplify in one-third of the samples (three of nine) due to increased competition for nucleotides and primers.

Results compiled from a total of 88 qRT-PCRs demonstrated that the average C_T for 100 copies of the CIPC was 34.7 (±1.1). For comparison, in the no-template-control reactions, where there was no competition with the enteroviral standard, the average CIPC C_T was 34.2 (±0.5, Fig. 2). Preliminary experiments using 1,000 CIPC copies per reaction caused a truncation in the dynamic range of the enteroviral standard assay, whereas 10 copies per reaction failed to amplify consistently, making it impossible to distinguish true inhibition from nonamplification (data not shown).

The amplification efficiency and slopes of the enteroviral standard curve with or without the CIPC additions were not statistically different ($P < 0.05$, Fig. 3 [as determined by analysis of variance]). The CIPC did not appear to reduce the sensitivity or reproducibility of enteroviral standard detection. The results from 86 reactions (nine enteroviral standard curves), compiled into one cumulative enteroviral standard curve show high linearity with an average qRT-PCR amplification efficiency of 0.91 (±0.18, Fig. 4).

The dynamic range of our qRT-PCR assay is wide (3 logs), ranging from 25 to 25,000 copies of the enteroviral standard (Fig. 3). However, at less than 250 copies the assay variability increases, with an overall average amplification failure at 25 enteroviral copies of 22%. This variability is indicated by the larger standard deviations in the enteroviral C_T values as the concentration of the enteroviral standards decreases (Fig. 4). Consequently, all standards were run in triplicate.

Results from the inhibition assay indicate that all of the extracted RNA samples possessed some level of qRT-PCR inhibitors present in the extracted RNA. As filtration volumes

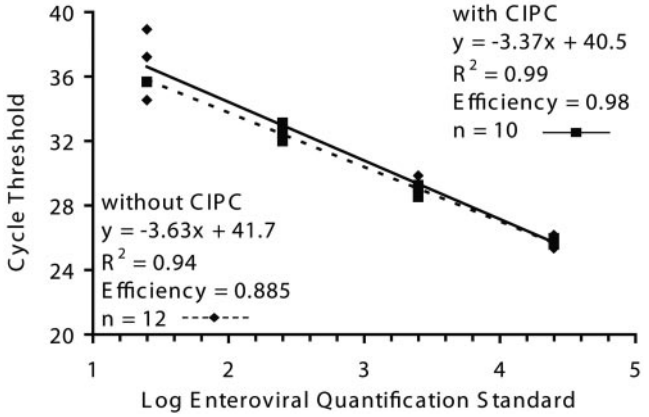

FIG. 3. Comparison of individual enteroviral standard curves with CIPC (■) or without CIPC (◆), demonstrating consistency among standard curves. The amplification efficiencies were calculated from the slopes of the linear regressions.

increased, so did the capture of inhibitory compounds, which resulted in a significantly reduced enteroviral standard detection (Table 2). The use of the CIPC as a calibrator reduced the effects of the inhibitors on enteroviral quantification and allowed a relatively accurate assessment of the level of enteroviral contamination in a given sample (≥75%) despite the presence of inhibitors (Table 2).

Minimum detection limits in PFU for the echovirus 12 stock. The titered echovirus 12 stock yielded an estimate of 102 (±11) detectable echovirus particles PFU^{-1} using the SYBR Green I method and 326 enteroviral quantification standard transcripts PFU^{-1} using qRT-PCR. These translate into lower limits of detection of 0.25 and 0.08 PFU based on the echovirus and enteroviral standard estimates using the lower limit of detection for the enteroviral qRT-PCR assay of 25 enteroviral transcripts. These data also indicate 3.2 (±0.7) genomes per stainable echovirus particle, which is higher than the expected 1:1 ratio based on the fact that enteroviruses contain one genome per virion.

Analysis of field samples. Upon reanalysis of the inhibited California field samples, 4 of the 27 (14.8%) samples were shown to contain amplifiable enteroviral genetic material, with concentrations ranging from 193 to 5.193 genomes $liter^{-1}$ (Table 3). Two of the extracts remained inhibited at this dilution—one completely and one partially (Table 3).

DISCUSSION

The qRT-PCR assay developed in this study can detect a broad range of human enteroviruses and can be used to detect human fecal contamination in environmental water samples. The assay is rapid and sensitive and allows a realistic assessment of PCR inhibition, an important feature for assays of real-world samples. It can be completed in less than 5 h from the sample to the result and is capable of detecting as few as 25 enteroviral genomes (Fig. 1 to 4). The assay can be used to screen recreational and shellfish-harvesting waters for human fecal contamination, thereby providing public health officials with a reliable assessment of the potential health risks associated with using these waters for recreation or shellfishing.

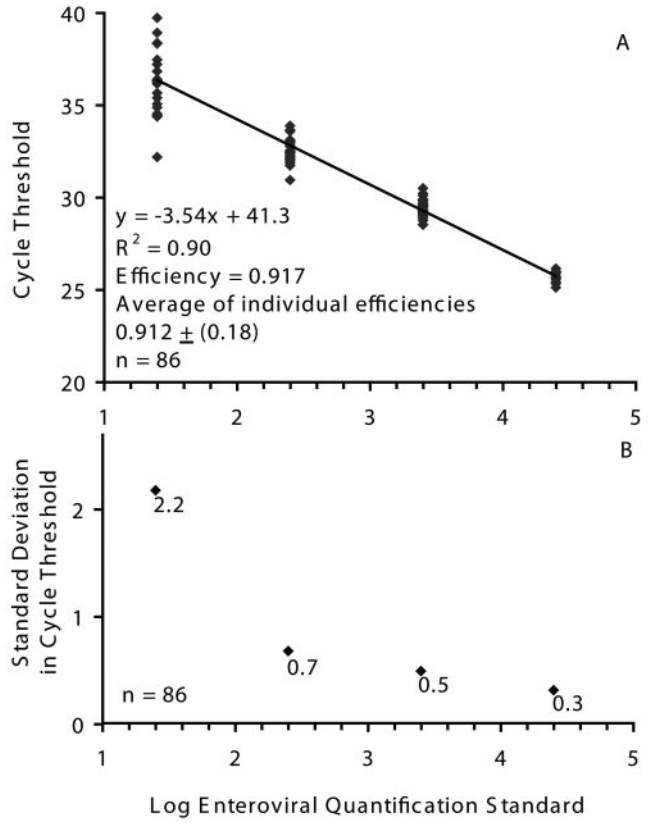

FIG. 4. (A) Cumulative enteroviral standard curve compiled from 86 reactions (nine individual enteroviral standard curves), amplified with the CIPC. The amplification efficiency was calculated from the slope of the linear regression. (B) Variability according to enteroviral quantification standard concentration is shown by plotting enteroviral qRT-PCR C_T as a function of concentration of enteroviral quantification standards. One standard deviation in C_T is represented by each point (◆) for the given log-transformed enteroviral quantification standard concentration.

One of the primary obstacles in developing qRT-PCR viral assays of this type for screening environmental samples is the isolation of inhibitors along with RNA during the extraction process. This has led to the use of adjuvants, such as PVP (14, 18), in the extraction process. The PVP binds various inhibitors and is easily separated during the RNA extraction process, helping prevent carryover of the inhibitors (2). Bovine serum albumin (15) and PVP have also been added directly to qRT-PCRs to bind inhibitors, thereby preventing them from interfering with the RT and DNA polymerase enzymes. Although these and other techniques have allowed significant progress in reducing the purification and suppressive effects of inhibitory compounds, the problem of inhibitors has not been eliminated. This necessitates the incorporation of an internal control to monitor inhibition. Otherwise, qRT-PCRs that failed due to inhibition cannot be distinguished from samples where viral loads were below the limits of detection. Protocols utilizing internal controls to monitor RT-PCR inhibition have been developed for enteroviral detection in clinical settings (17), but few have been developed for use in the amplification of environmental samples (23).

TABLE 2. Deep Creek, Beaufort, N.C., water sample RNA extracts analyzed with the enteroviral qRT-PCR assay[a]

Environmental extraction	Enteroviral FAM C_T	CIPC Cy5 C_T	Enteroviral concn without ΔCIPC C_T	Avg enteroviral concn without ΔCIPC C_T	Avg % of enteroviral standard detected without ΔCIPC C_T	ΔCIPC C_T	Adjusted enteroviral concn with ΔCIPC C_T	Avg % of enteroviral standard detected with ΔCIPC C_T
100 ml	33.52	36.16	191	317	29.9	1.57	859	81.0
100 ml	32.20	36.19	442					
250 ml	34.84	38.40	83	139	13.1	2.75	796	75.1
250 ml	33.50	36.31	194					
400 ml	35.36	39.79	60	84	7.9	3.81[b]	943	89.0
400 ml	34.42	37.04	108					
No extract	30.78	34.07	1,086	1,060	100.0		1,060	
No extract	30.86	35.14	1,033					
Avg CIPC C_T neg controls		34.61						

[a] A total of 1,000 copies of the enteroviral quantification standard and 100 copies of the CIPC were added directly to the qRT-PCR master mix. Enteroviral concentrations were calculated by using the enteroviral standard curve with or without the ΔCIPC C_T calculation. Control reaction mixtures containing none of the extract from the Deep Creek samples were run in parallel to estimate the accuracy of the enteroviral assay. neg, negative.

[b] Under normal circumstances, this sample would have met the criteria for inhibition and would have been diluted and reanalyzed.

The assay developed in the present study incorporated a CIPC in each sample to assess inhibition. The CIPC contained the same conserved primers sites as utilized for amplifying the target enteroviral sequence, and a sequence of similar size and composition to the target enteroviral template (CIPC amplicon [53% GC] and enteroviral amplicon [54% GC]). Amplification of the CIPC and enteroviral templates was distinguished by utilizing a different TaqMan probe specific to each template. Assay development further required an empirical determination of the minimal CIPC concentration that produced a consistent amplification without significantly reducing the sensitivity, specificity, or reproducibility of the enteroviral assay. Preliminary studies showed that 100 CIPC copies per reaction amplified reliably at a C_T of between 34.5 and 35.0 over a range of 25 to 25,000 standard enteroviral transcripts per reaction (Fig. 1 and 2). At high enteroviral transcript concentrations (e.g., >25,000 enteroviral transcripts per reaction), however, the CIPC failed to amplify in 33% of the samples. These CIPC failures were probably due to competition between the CIPC and enteroviral quantification standard for nucleotides and primers in the qRT-PCRs. When analyzing field samples, these CIPC failures are likely to be rare because reported environmental enteroviral RNA concentrations measured to date fall far below the levels that would cause a problem (see, for example, references 4, 9, and 21). If samples with enteroviral transcripts per reaction above 20,000 to 25,000 were to be encountered, they should be diluted and reanalyzed to ensure accurate enteroviral estimates, or the researcher can choose to reoptimize the CIPC concentration for use with higher concentrations of target.

TABLE 3. Results from Ballona Creek, Calif., field samples analyzed with the enteroviral qRT-PCR[a]

Field sample	Initial analysis		Postdilution analysis		
	ΔCIPC C_T	Dilution	ΔCIPC C_T	Genomes liter^{-1}	Adjusted genomes liter^{-1}
8	>11	1:2	1.2	0	0
9	>11	1:2	0.88	0	0
13	>11	1:2	1.11	0	0
19	>11	1:2	1.6	0	0
20	>11	1:2	1.05	0	0
21	>11	1:2	2.13	0	0
23	>11	1:2	0.59	0	0
28	>11	1:2	1.57	1,641	4,507
29	>11	1:2	3.41	579	5,193
30	>11	1:2	1.67	0	0
31	>11	1:2	0.66	0	0
32	>11	1:2	0.75	0	0
33	>11	1:2	2.95	0	0
34	>11	1:2	3.92[b]	0	0
39	9.48	1:2	3.34	0	0
41	10.07	1:2	3.21	0	0
43	>11	1:2	2.55	0	0
44	4.92	1:2	0.46	0	0
45	>11	1:2	>11[c]	0	0
46	>11	1:2	1.79	61	193
47	>11	1:2	1.02	0	0
48	>11	1:2	2.71	384	2,193
51	>11	1:2	1.1	0	0
53	>11	1:2	1.11	0	0
54	>11	1:2	0.87	0	0
58	>11	1:2	1.5	0	0
59	6.81	1:2	3.25	0	0

[a] Inhibition was determined by subtracting the average CIPC C_T (34.6) from the sample CIPC C_T. Samples in which the ΔCIPC was less than 3.7 C_T were considered not to have inhibition, and genome concentrations were adjusted using the ΔCIPC C_T as a calibrator. Samples with a ΔCIPC C_T of between 3.7 and 11 were considered partially inhibited, and samples with ΔCIPC C_T values of >11 were considered completely inhibited. In samples positive for enterovirus, the genomes liter^{-1} were determined by using the calculated genomes reaction^{-1} extrapolated to the total volume analyzed, taking into account dilution and scaling the number up to 1 liter.

[b] Sample still partially inhibited after dilution.

[c] Sample still completely inhibited after dilution.

The ability of the CIPC to identify inhibited samples was also readily demonstrated in a recent field study where, despite careful extraction procedures, 61% of the qRT-PCRs using this assay were found to be significantly inhibited (21). In the present study, we used a subset (n = 27) of the inhibited samples to investigated whether a simple 1:2 dilution of with RNase-free water would reduce the PCR inhibition sufficiently for reliable enteroviral estimates to be obtained. Upon reanalysis, 4 of the diluted samples contained measurable concentrations of enteroviruses, and 21 were negative for enteroviruses, but no longer showed significant inhibition. This demonstrated the utility of using the CIPC and simple dilution methods to identify inhibited samples and to differentiate false negatives from samples with enteroviral concentrations below assay detection levels.

Within limits, the CIPC can also be used to successfully correct enteroviral estimates obtained from partially inhibited

qRT-PCR samples. This is accomplished by using the difference in CIPC C_T values between control and field samples, in conjunction with the qRT-PCR amplification efficiency, to calculate the fold reduction in the CIPC for each sample. The CIPC fold reduction for each sample is then used to correct the actual enteroviral estimate to that which would have been obtained if no inhibition had occurred. Reasonable enteroviral concentrations can then be back-calculated for samples showing up to ~90% inhibition (ΔCIPC C_T of ~3.7). Employing this method, we were able to adjust enteroviral concentrations, in our spiked samples, to within 75% of their expected values (Table 2).

Although the dilution scheme worked in most cases to eliminate inhibition (Table 3), it has the obvious disadvantage that, at very low enteroviral levels, the chance exists that the dilution itself will take the sample below levels of detection. With small dilutions, such as those used in the present study, the chance of losing signal solely on the basis of dilution is minimized. Optimally, improvements in viral filtration and RNA extraction methods will reduce qRT-PCR inhibitor levels in reactions, but without adequate internal controls for the qRT-PCR the effectiveness of these new methods cannot be validated.

The data obtained in validating this assay indicate that as the enteroviral loads decrease, assay variability increases dramatically (Fig. 4). Given these data, it would be logical to assume that the best strategy would be to filter more environmental sample to obtain higher viral concentrations in order to reduce assay variability. This approach, however, proved counterproductive. For sample sizes greater than 250 to 500 ml, the increased coisolation of inhibitors completely negated any gains in amplification efficiency that was achieved by isolating a higher concentration of viral genomes (Table 2). By reducing filtration volumes, the capture of inhibitory compounds can be minimized, improving the RNA extraction and subsequent qRT-PCR and, through the use of the CIPC as a calibrator, any effects from inhibition can be effectively corrected for. Future improvements in eliminating elements inhibitory to the qRT-PCR or in selectively removing viral genomes from bulk samples will be required before large-volume samples from most recreational waters can be quantitatively assayed. In the interim, accurate estimates of low enteroviral concentration (<50 genome equivalents per sample) will require analysis of multiple replicates per sample.

Relating results obtained from qRT-PCR assays to levels of infectious virus is vital for the assessment of public health risk and epidemiological models. Any detection of enteroviral genetic material is indicative of the presence of enteroviruses, but the actual abundance of infectious enteroviral particles is harder to elucidate. Previously, PFU have been used as a measure of infectious particles, but it is likely that a PFU greatly underestimates the number of actual infectious virions in a sample because of the inherent nature of PFU-based assays. In the present study, we estimated that the lower limit of detection for the qRT-PCR assay was somewhere between 0.08 and 0.25 PFU for echovirus 12. Although this lower range of sensitivity is likely to vary from enterovirus to enterovirus and from host to host, the echovirus 12 results demonstrate that the assay is capable of measuring clinically relevant levels of enteroviruses in environmental samples.

Accurate quantification of human enteroviral genomes in environmental waters is necessary to assess potential public health risk associated with water contact and also for tracking the sources of human fecal contamination (21). The enteroviral qRT-PCR assay developed in the present study is rapid and can be applied to any study requiring accurate quantification of enteroviruses in environmental samples. The dynamic range of the assay was 3 logs, from 25 to 25,000 genome equivalents. As with any PCR-based assay for viral pathogens, it is limited by only being able to detect genomes rather than to directly assess infectivity. However, control studies with echovirus 12 virus indicate that, while not measuring infectivity directly, the assay sensitivity does allow clinically relevant levels of enteroviruses (in the 0.08- to 0.25-PFU range) to be detected. The assay incorporated a CIPC that allowed rapid identification of inhibited samples. In a majority of cases, inhibition was reduced sufficiently by diluting samples, so that false negatives could be distinguished from samples with enteroviral concentrations below detection levels. The CIPC was also used to estimate correction factors for enteroviral estimates obtained from partially inhibited samples, thereby allowing improved estimates of enteroviral loads in complex environmental water samples. The improved accuracy provided by the CIPC represents a major advancement in the field. Application of this assay will make it possible to accurately assess enteroviral loads in both recreational and shellfish-harvesting waters, hopefully providing improved means for management and restoration of these resources.

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