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A highly sensitive method for detecting *Cryptosporidium parvum* oocysts recovered from source and finished water using RT-PCR directed to *Cryspovirus* RNA

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ABSTRACT

Sensitive detection of *Cryptosporidium* oocysts is important because the protozoan can cause clinical infection in humans at extremely low numbers. In the present study, 1.5×10^2, 1.0×10^3, or 1.0×10^4 *C. parvum* oocysts were spiked into 10 l of source or finished water in triplicate followed by recovery using Envirochek HV sampling capsules. One subsample of the recovered oocysts was analyzed by commercial immunofluorescence assay (IFA), while a second subsample was subjected to DNA-RNA extraction, followed by RT-PCR using primers directed to the gene encoding *Cryspovirus* capsid. IFA analysis of Envirochek filter eluates of finished water detected oocysts at all 3 *C. parvum* oocyst doses, but only at the 1.0×10^3 and 1.0×10^4 doses in source water. *Cryspovirus* RT-PCR appeared to offer greater sensitivity than IFA because *C. parvum* oocysts were detected using this molecular technique in both source and finished water concentrates at all 3 spiking levels. A linear relationship was observed between log oocysts spiking dose and the relative intensity of the *Cryspovirus* RT-PCR signal for finished water, but not for source water. These data indicate that *Cryspovirus* RT-PCR is a sensitive method for detecting *C. parvum* oocysts in source and finished water.

1. Introduction

Cryptosporidiosis is an important waterborne disease. Outbreaks of cryptosporidiosis continue to regularly affect communities, especially those in developing countries (for review see Shirley et al., 2012). *Cryptosporidium* is particularly resistant to environmental decay and to chlorination processes in water treatment plants, thus requiring additional disinfection steps such as exposure to UV irradiation or ozonation (King et al., 2016). The low $I.D._{50}$ of *C. parvum* for humans (as few as 10 oocysts depending on strain) (Okhuysen et al., 1999; Messner et al., 2001; Chappell et al., 2006) and its resilience to disinfection is the reason that routine monitoring of source water for the protozoan is conducted in many countries (for review see Carey et al., 2004). The EPA Method 1623 describes a procedure for testing source water for the presence of *Cryptosporidium* oocysts and *Giardia* cysts using commercial immuno-fluorescence assay (IFA, McCuin and Clancy, 2003). While this method is adequate for detecting high numbers of oocysts and cysts in water, it suffers from low sensitivity and an inability to differentiate between species infectious and not infectious for humans. Several molecular approaches involving PCR directed to different gene targets have been described and are capable of detecting extremely low numbers of *Cryptosporidium* in a water sample (for review see Kothavade, 2012; Adeyemo et al., 2018). The targets include genes encoding small subunit (SSU) ribosomal RNA (Li et al., 2015), *Cryptosporidium* oocyst wall protein (Guy et al., 2003; Alonso et al., 2014), heat-shock protein 70 (Monis and Saint, 2001; Ware et al., 2013) and heat-shock protein 90 (King et al., 2016). Detection of *Cryptosporidium* oocysts using these targets include single and nested PCR (Ruecker et al., 2013; Alonso et al., 2014), multiplex PCR (Rubio et al., 2014), TaqMan assay incorporating minor groove binding probes (Burnet et al., 2013) and real-time PCR (qPCR; Liang and Keeley, 2012; Moss et al., 2014). A few authors have incorporated additional steps to differentiate viable from non-viable oocysts using vital stains (Alonso et al., 2014; Liang and Keeley, 2012) as well as random fragment length polymorphism analysis and DNA sequencing to further ascertain *Cryptosporidium* species, genotypes, and subtypes (Leav et al., 2002; Xiao et al., 2004; Abe et al., 2006; Li et al., 2015).

An approach that our group and others have taken is to utilize

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dsRNA associated with the viral symbiont *Cryspovirus*, that is present in *Cryptosporidium* species infecting humans, including *C. parvum*, *C. hominis*, *C. felis*, and *C. meleagridis* (Leoni et al., 2006; Nibert et al., 2009) to detect low numbers of the protozoan in environmental samples (Kozwich et al., 2000; Jenkins et al., 2016). Higher sensitivity of *Cryspovirus*-based RT-PCR assay is probably due to the higher copy number of dsRNA molecules in *Cryptosporidium* (5000/oocyst) (Khramtsov et al., 1997) compared to gene targets such as 18S rDNA (20 copies/oocyst)(Abrahamsen et al., 2004; Li et al., 2015). While *Cryspovirus*-based RT-PCR assays have shown promise in detecting *C. parvum* in cell culture (Jenkins et al., 2015) and human feces (Murakoshi et al., 2016), no one has applied this methodology to large volumes (≥10 l) of finished or source water. The purpose of this study was to evaluate the utility of a *Cryspovirus* RT-PCR for the detection of *Cryptosporidium* oocysts recovered from oocysts-spiked finished and source water using the approved EPA Method 1623.

2. Materials & methods

A series of oocyst spiking studies were conducted to evaluate the usefulness of *Cryspovirus* RT-PCR for detecting *C. parvum* oocysts in water. Source water (100 l) was obtained at a single timepoint from the Washington Suburban Sanitary Commission (WSSC) Patuxent Water Filtration Plant which is supplied water from the Triadelphia reservoir, a highly stable water quality source (Robert Buglass, personal communication). Water quality parameters for the source water were as follows: alkalinity – 32 mg/l, dissolved oxygen – 3.4 mg/l, turbidity – 5.4 NTU, pH – 7.0, temperature – 27.7 °C. Source water treatment involved standard water treatment processes in the following order-chemical treatment with polyaluminum chloride, coagulation, flocculation, sedimentation, chlorination, deep bed filtration with anthracite/granulated activated carbon media, addition of fluoride, lime, chlorine and orthophosphate. Finished water (100 l, NTU = 0.03) was obtained at a single timepoint from a residential tap receiving water from the WSSC Patuxent Plant. Source and finished water testing prior to spiking studies for *Cryptosporidium parvum* oocysts using the methods described below failed to detect the protozoan. *Cryptosporidium parvum* (Iowa strain) oocysts were obtained from the University of Arizona and used within 6 months of propagation in dairy calves. Oocysts were propagated and purified by CsCl centrifugation at the University of Arizona using standard procedures (Arrowood and Donaldson, 1996). The concentration of *C. parvum* oocysts used in the spiking studies was confirmed by pipetting 10 µl of the suspension in triplicate onto 3-well treated microscope slides, drying the slides at room temperature, and immunolabeling using MeriFluor *Cryptosporidium/Giardia* reagents and instructions provided by the manufacturer (Meridian Biosciences, Inc., Cincinnati, OH). A 1 ml volume equivalent to 1.5×10^2, 1.0×10^3, or 1.0×10^4*C. parvum* oocysts were introduced by pipetting directly into source or finished water (10 l/experiment) in 25 l plastic carboys in triplicate, followed by thorough mixing. Prior to oocyst spiking, 0.2 g silica gel (250–500 µm particle size, Sigma Chemical Co., St. Louis, MO) was added to the 10 l water sample to improve oocyst recovery using a modification described elsewhere (Feng et al., 2003). The 10 l water-oocyst mixture was passed through an Envirochek HV capsule (Pall Corporation, Port Washington, NY) using a Masterflex I/P pump and controller (Cole-Parmer, Vernon Hills, IL) at 2.0 l/min flow rate. Elution and concentration of *C. parvum* oocysts from the Envirochek HV capsule was performed following manufacturer's instructions (Pall Corporation). After a final centrifugation step, the entire water sample concentrate was suspended in 10 ml deionized water, silica and detrital material was allowed to settle for 5–10 min, and the entire overlying suspension was transferred to a 15 ml polypropylene test tube. The oocysts were pelleted by centrifugation for 10 min at 3000*g* and suspended in 2 ml deionized H_2O for oocyst counting and extraction of DNA/RNA for RT-PCR analysis.

Aliquots (10 µl) of the suspension were transferred to individual wells of a 3-well microscope slide and allowed to air-dry. Detection of *C. parvum* oocysts was achieved using a Merifluor *Cryptosporidium/Giardia* immunofluorescence kit (Meridian Biosciences, Inc.) as described above. Enumerating *C. parvum* oocysts was accomplished using a Zeiss Axioskop 2 microscope under 400× magnification. Average counts were calculated for the triplicate wells, and used to calculate mean and S.E. for triplicate spiking studies done at each *C. parvum* oocyst inoculation dose.

An aliquot (0.5 ml) from the same 2 ml suspension was removed to a 1.5 ml microcentrifuge tube, followed by centrifugation at 3000*g* for 5 min. to pellet oocysts and detrital material. The supernatant was carefully removed, and the pellet (~100 µl in size originating from source water) was subjected to a DNA/RNA extraction using a Qiagen Stool DNA Extraction kit following manufacturer's procedures with minor modifications (Qiagen, Hilden, Germany). The advantage of using the DNA extraction kit is that both DNA and viral RNA are recovered, the latter capable of being detected by RT-PCR (Jenkins et al., 2016). The only modifications to the procedure were the use of 180 mg (~½ tablet) instead of 370 mg InhibitEX (whole tablet), the entire overlying supernatant removed after spinning down the InhibitEX, and a final EtOH precipitation of the final column eluate. In previous studies conducted in our laboratory, it was found that using less InhibitEX produced greater DNA recovery without compromising the removal of PCR inhibitory material (unpublished observation). The concentration and purity of nucleic acids in the sample was estimated by reading $O.D._{260}$ and $O.D._{260/280}$ respectively on a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). Duplicate RT-PCR directed at the *Cryspovirus* capsid RNA sequence was employed to analyze about 5 ng RNA using described procedures (Jenkins et al., 2015). In brief, RT-PCR involved denaturing dsRNA in the presence of *Cryspovirus* capsid forward (CPV-F, 5′ TGGTTCCGATTTTACCGGAA 3′) and reverse (CPV-R, 5′ ACGACAATTAGGACTCAAATGACC 3′) primers in a boiling water bath for 1 min followed by immersion in an ice bath slurry. RT-PCR utilized the Superscript III One-Step RT-PCR system (Invitrogen) and was carried out in a PTC100 thermocycler (MJ Research, Watertown, MA) using the following conditions: 47 °C, 30 min., denaturation at 94 °C, 3 min., followed by 35 cycles of 94 °C, 30 s; 50 °C, 30 s; 72 °C, 1 min, followed by a final extension at 72 °C, 5 min. An internal DNA standard (competitor) comprised of an irrelevant DNA sequence flanked on the ends with either CPV-F or CPV-R primer sequence was included in all RT-PCR reactions (see Ross et al., 1995; Jenkins et al., 2016). The purpose of the internal standard/competitor is to control for false negative results due to the presence of PCR inhibitors. A negative control (H_2O only) was included in each set of reactions to control for *Cryspovirus* RNA contamination. The RT-PCR amplicons (target ~350 bp, competitor ~500 bp) were analyzed by electrophoresis on 7.5% polyacrylamide gels (Sambrook et al., 1989) followed by image capture on a Kodak Image Capture device and densitometry analysis using AlphaView SA software (ProteinSimple.com). A target:competitor ratio (T:C) was calculated from all densitometry readings for each RT-PCR. Mean and S.E. values were calculated for each triplicate sample, and examined for a linear relationship between log oocyst spiking dose and T:C ratio using GraphPad Instat (GraphPad Software, San Diego, CA). Preliminary analysis of source and finished water samples by IFA and RT-PCR prior to oocyst spiking revealed negligible levels of *C. parvum*.

3. Results and discussion

3.1. Source water

Using IFA to detect *Cryptosporidium* oocysts recovered from *C. parvum* oocyst-spiked source water identified the parasite in 3/3 concentrated samples at the 1.0×10^4 spiking dose and 2/3 samples at the 1.0×10^3 spiking dose. IFA failed to detect any *C. parvum* oocysts in water concentrates from the 1.5×10^2 oocyst spiking dose. Percentage

Table 1
Detection of *Cryptosporidium parvum* oocysts recovered using EPA Method 1623 from 10 l source or finished water spiked with 1.5×10^2, 1.0×10^3, 1.0×10^4 *C. parvum* oocysts and analyzed using commercial Merifluor *Cryptosporidium/Giardia* staining kit ($n = 3$ replicates for each spiking dose-water type combination).

Number *C. parvum* oocysts inoculated into water	Percentage Recovery[a]	
	Source Water	Finished Water
1.0×10^4	52 ± 15	47 ± 5
1.0×10^3	70 ± 51	50 ± 14
1.5×10^2	0	45 ± 31

[a] Percentage recovery was calculated as the number of oocysts recovered divided by the number of oocysts inoculated into 10 l source or finished water.

Table 2
Detection of *Cryptosporidium parvum* oocysts recovered using EPA Method 1623 from 10 l source or finished water spiked with 1.5×10^2, 1.0×10^3, 1.0×10^4 *C. parvum* oocysts and analyzed using *Cryspovirus*-specific RT-PCR (n = 3 replicates for each spiking dose-water type combination).

Number *C. parvum* oocysts inoculated into water	Mean target:Competitor ratio[a]	
	Source Water	Finished Water
1.0×10^4	0.37 ± 0.24	3.4 ± 0.80
1.0×10^3	0.09 ± 0.06	1.2 ± 0.10
1.5×10^2	0.22 ± 0.14	0.27 ± 0.08

[a] Target:Competitor Ratio is calculated from densitometry value of RT-PCR amplification of *Cryspovirus* RNA target divided by densitometry value of internal standard (competitor).

recovery estimated by dividing the number of *C. parvum* oocysts recovered in IFA-positive samples by the number of oocysts inoculated into 10 l source water was 52% at the 1.0×10^4 spiking dose and 70% at the 1.0×10^3 spiking dose (Table 1). *Cryspovirus* RT-PCR detected *C. parvum* oocysts in all 3 source water samples at each spiking dose including the 1.5×10^2 level suggesting that it is more sensitive than IFA (Table 2). However, no linear relationship was observed between the target:competitor (T:C) ratio intensities and log oocyst spiking dose for source water ($r^2 = 0.35$, $P > .05$, Table 2).

3.2. Finished water

Using IFA to detect *Cryptosporidium* oocysts recovered from *C. parvum* oocyst-spiked finished water identified the parasite in 3/3 concentrated samples at both the 1.0×10^4 and the 1.0×10^3 spiking doses. Unlike source water, IFA detected *C. parvum* oocysts in 2/3 concentrated samples originating from the 1.5×10^2 spiking dose. Percentage recovery estimated by dividing the number of *C. parvum* oocysts recovered in IFA-positive samples by the number of oocysts inoculated into 10 l finished water was 47% at the 1.0×10^4 spiking dose, 50% at the 1.0×10^3 spiking dose, and 45% at the 1.5×10^2 spiking dose (Table 1). RT-PCR also detected *C. parvum* in all finished water samples inoculated with 1.5×10^2, 1.0×10^3, or 1.0×10^4 oocysts. Moreover, a linear increase in T:C ratio was observed with increasing number of *C. parvum* oocysts inoculated into finished water ($r^2 = 0.98$, $P < .05$, Table 2).

Greater inhibition of RT-PCR was apparent with source water samples compared to finished water, which is probably not unexpected and may explain the lack of relationship between oocyst dose and T:C ratio in source water samples. Although adding silica gel to both source and finished water improved oocyst recovery, incorporating a secondary immunomagnetic separation (IMS) step failed to improve recovery of *C. parvum* oocysts. While IMS has been used for increasing the recovery of *Cryptosporidium* and *Giardia* from source water (Deng et al., 2000; Hallier-Soulier and Guillot, 2003), others have found minimal improvement in oocyst and cyst recovery (Maciel and Sabogal-Paz, 2016). It is possible that particulates in the source water used in the present study interfered with binding of *C. parvum* oocysts to the IMS beads, as has been noted by others (Kuhn et al., 2002; Bukhari et al., 1998).

Although Cryspovirus RT-PCR appeared to offer greater sensitivity than IFA in detecting *C. parvum* oocysts recovered from source water spiked with 150 oocysts, the variation in recovery of oocysts at all spiking doses was similar between the two detection methods. This variation has been observed by others conducting similar spiking studies using IFA or molecular methods, suggesting that the water concentration step rather than method of detection is responsible for the observed variation in recovery (Mayer and Palmer, 1996; Kostrzynska et al., 1999; Chesnot et al., 2002; Ongerth and Saaed, 2013).

4. Conclusions

This study provides an extremely sensitive method for detecting *C. parvum* oocysts that have been recovered from source and finished water using the EPA Method 1623. The RT-PCR utilizes primers that are useful in amplifying the *Cryspovirus* capsid-encoding gene from various species of *Cryptosporidium* infectious for humans. This method could be used as a water screening method, and once a positive sample is identified, subsequent molecular methods can be applied to the DNA/RNA sample for genotyping the oocysts and estimate the threat to human health and for subtyping to possibly determine sources of infection (Xiao et al., 2004; Abe et al., 2006; Ruecker et al., 2013).

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