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S. D. Sluter · S. Tzipori · G. Widmer

# Parameters affecting polymerase chain reaction detection of waterborne *Cryptosporidium parvum* oocysts

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**Abstract** *Cryptosporidium parvum* is an enteric protozoan parasite of medical and veterinary importance. Dissemination of environmentally resistant oocysts in surface water plays an important role in the epidemiology of cryptospridiosis. Although the polymerase chain reaction (PCR) is a well-established technique and is widely used for detecting microorganisms, it is not routinely applied for monitoring waterborne *C. parvum*. In order to facilitate the application of PCR to the detection of waterborne *C. parvum* oocysts, a comparison of published PCR protocols was undertaken and different sample-preparation methods tested. The sensitivity of a one-step PCR method, consisting of 40 temperature cycles, was 10 purified oocysts or fewer than 100 oocysts spiked in raw lake water. The detection limit of two primer pairs, one targeting the ribosomal small subunit and another specific for a *C. parvum* sequence of unknown function, was approximately ten-fold lower than achieved with a primer pair targeting an oocyst shell protein gene. Three cycles of freezing/thawing were sufficient to expose oocyst DNA and resulted in higher sensitivity than proteinase K digestion, sonication or electroporation. Inhibition of PCR by surface water from different local sources was entirely associated with the soluble fraction of lake water. Membrane filtration was evaluated in bench-scale experiments as a means of removing lake water inhibitors and improving the detection limit of PCR. Using gel and membrane filtration, the molecular size of inhibitory solutes from lake water was estimated to less than 27 kDa.

S. D. Sluter
Department of Biology and Biotechnology,
Worcester Polytechnic Institute, Worcester, MA 01609, USA

S. Tzipori[1] · G. Widmer (✉)
Division of Infectious Diseases, Department of Comparative Medicine, Tufts University School of Veterinary Medicine,
Building 20, North Grafton, MA 01536, USA
Tel.: (508) 839 7944
Fax: (508) 839 7977
e-mail: gwidmer@opal.tufts.edu

[1] *Also*: Division of Geographic Medicine,
New England Medical Center, Boston, MA 02111, USA

## Introduction

*Cryptosporidium parvum* is a widespread coccidian parasite causing diarrhea in humans and susceptible animals. Whereas in the immunocompetent host the infection is typically self-limiting, cryptosporidiosis in immunosupressed individuals is often chronic and can be life-threatening (Ungar 1990). Recent outbreaks of cryptosporidiosis caused by drinking water contaminated with oocysts (Solo-Gabriel and Neumeister 1996; Widmer et al. 1996), and the frequent occurrence of *C. parvum* oocysts in untreated surface water (LeChevallier and Norton 1995; Ongerth and Stibbs 1987; Rose et al. 1991; Smith and Rose 1990) has highlighted the need for methods capable of reliably detecting low concentrations of oocysts in raw and finished water. Because of its sensitivity and specificity, the polymerase chain reaction (PCR) is ideal for detecting environmental microorganisms including waterborne oocysts. An optimized PCR protocol could have significant advantages over the commonly used immunofluorescence detection, which is labor-intensive and requires the interpretation of fluorescent microscopy images in the presence of large number of debris and fluorescent signals caused by cross-reacting microorganisms (Rodgers et al. 1995).

A number of PCR protocols for the selective detection of *C. parvum* have been published (Carraway et al. 1996; Johnson et al. 1995; Laxer et al. 1991; Morgan et al. 1996; Ranucci et al. 1993; Webster et al. 1993) but these protocols have not been compared with respect to their sensitivity. For some of these methods the targeted genetic locus is known (Carraway et al. 1996; Johnson et al. 1995; Ranucci et al. 1993), whereas other published PCR primers amplify unknown genomic regions (Laxer et al. 1991; Morgan et al. 1996; Webster et al. 1993). As

the PCR signal intensity is strongly affected by a variety of factors, foremost the copy number of the target and the efficiency of the primers, a direct comparison of different PCR protocols is warranted as a first step towards the optimization of PCR detection of *C. parvum*.

Thermostable DNA polymerases, commonly used to drive PCR amplification, are known to be inhibited by environmental factors. This inhibitory activity has been attributed to humic substances (Tebbe and Vahjen 1993; Tsai and Olson 1992). Consistent with these observations is that physical separation of oocysts from environmental samples by magnetic-bead capture or flow cytometry resulted in enhanced recovery (Johnson et al. 1995).

In this study, different published PCR primers were evaluated and their sensitivity compared, with the aim of optimizing PCR detection of *C. parvum*. Substances inhibitory to PCR amplification were found seasonally in local lake and creek water. Membrane and gel filtration was used to characterize the solubility and molecular mass of PCR-inhibiting substances. A membrane filtration method was developed that enhanced the sensitivity of PCR detection. The sensitivity achieved by this method was similar to that observed with purified oocysts.

## Materials and methods

### Water samples

Water was collected in plastic containers at two locations: Lake Chauncy, Westboro, Mass., and a small creek located in an urbanized area of Westboro. Water samples were stored at 4 °C.

### Oocysts and oocyst disruption

*C. parvum* oocysts of isolates GCH1, UCP (Tzipori et al. 1994), and MD (Lally et al. 1992) were propagated in neonatal calves at the Tufts University *Cryptosporidium* core facility in North Grafton, Mass. Purification was performed according to Arrowood and Sterling (1987). Oocysts were disrupted by three cycles of freezing/thawing in ethanol/solid $CO_2$ and a 37 °C waterbath. The following alternative disruption methods were attempted using suspensions of $10^2$ and $10^4$ oocysts in distilled water: (a) sonication for three periods of 5 minutes was performed in a sonicating waterbath (Branson, Danbury, Conn.); (b) proteinase K digestion (200 μg/ml), was carried out in the presence of 0.2% sodium dodecyl sulfate at 45 °C overnight. This was followed by phenol/chloroform extraction and DNA concentration by ethanol precipitation in 0.2 M sodium chloride.

### Polymerase chain reaction

PCR was performed on a Gradient 40 Robotcycler (Stratagene) in standard or thin-walled 0.5-ml microcentrifuge tubes. The reaction mix consisted of 1 × reaction buffer (Promega) supplemented with 2 mM $MgCl_2$ (unless otherwise indicated), 200 μM deoxynucleoside triphosphates, 400 nM each primer and approximately 2.5 units *Taq* polymerase (Promega). Unless otherwise stated, primers CRY4 and CRY2 were used (Carraway et al. 1996). The hot-start method was applied throughout (D'Aquila et al. 1991). A total of 30 or 40 cycles of 94 °C, 40 s; 55 °C, 50 s; 72 °C, 50 s were applied unless otherwise indicated. The optimal annealing temperature for selected primers was determined experimentally, following optimization of the $Mg^{2+}$ concentration, using the gradient function of the cycler. When testing for inhibition by raw water or filtrates, 38 μl raw water was spiked with $2 \times 10^4$ oocysts and frozen/thawed as described above, and 10 μl PCR premix was added.

### Fractionation of lake water

A portion of 50 ml lake water was filtered by vacuum suction through a 0.45-μm cellulose nitrate membrane filter (Nalgene) and the retentate (fraction retained in the filter) resuspended in distilled water to the original volume of 50 ml. The filtrate from the first step was subject to a second filtration through a 0.2-μm cellulose nitrate filter. The retentate from this second filtration step was again reconstituted to its original volume with distilled water. A portion of 2 ml filtrate was passed through a spin filter of 100-kDa cut-off (Centricon, Amicon). This step was followed by two analogous filtration steps through spin filters of 30 kDa and 10 kDa cut-off. Retentates were again resuspended in their original volumes. Portions of 38 μl of each resuspended retentate and filtrate were spiked with *C. parvum* DNA equivalent to approximately $10^4$ oocysts and PCR-amplified in standard 50 μl reactions.

Gel filtration was performed using a Bio-Gel P-60 (Bio-Rad) column of 46 cm length equilibrated in distilled water. A volume of 15 ml autumn lake water was concentrated to 1 ml in a vacuum concentrator (Speed-Vac, Savant) and the concentrated sample eluted from the column with water. Fractions of 4.5 ml were collected and concentrated to approximately 30 μl by vacuum concentration. A portion of 10 μl of every third fraction was spiked with oocyst DNA and assayed for PCR inhibition in a standard 50-μl PCR reaction using primers CRY44 (CTCTTAATCCAATC ATTACAAC) and CRY39 (GAGTCTAATAATAAACCACTG; Carraway, Tzipori and Widmer, 1997) and 30 cycles of 94 °C for 50 s, 55 °C for 1 min , 72 °C for 1 min. A column void volume of 21 ml was estimated with blue dextran and the column calibrated with ovalbumin, and ribonuclease A. Elution of protein standards was monitored at 280 nm.

### Oocyst titration

To estimate the effect of different retentate concentrations on PCR detection, aliquots of 10, 100 and 500 ml autumn lake water were filtered through 0.2-μm cellulose nitrate filters, washed with 10 ml distilled water and resuspended in 10 ml distilled water. Aliquots of 40 μl of resuspended retentates were spiked with $4 \times 10^4$, $4 \times 10^3$, $4 \times 10^2$ and 40 oocysts and tested in standard PCR reactions using 40 temperature cycles.

## Results

### Primer optimization and comparison

Primers LA1 and LA2, slightly modified from primer 1 and primer 2 (Laxer et al. 1991), primers "Forward" and "Reverse" (Webster et al. 1993), CRY4 and CRY2 (Carraway et al. 1996) and CRIS3 and CRIS6, based on the sequence of an oocyst wall protein (nucleotide positions 721–741 and 1849–1827 respectively) (Ranucci et al. 1993), were compared with respect to their suitability for detecting *C. parvum* DNA of different isolates (Table 1). The Forward and Reverse primers failed to detect DNA from isolates GCH1, UCP and Iowa (Arrowood and Sterling 1987) when tested at annealing temperatures ranging from 50 °C to 60 °C (not shown). Two alternative primers based on the same sequence

**Table 1** Comparison of polymerase chain reaction (PCR) primer pairs for the detection of *Cryptosporidium parvum* oocysts. + positive PCR detection, – no signal detected. Nucleotides represented by lower-case letters indicate added restriction sites. *SSU* small subunit

| Primer | Target | Sequence (5′ to 3′) | Signal | Reference |
|---|---|---|---|---|
| LA1 | Unknown | CCGAGTTTGATCCAAAAAGTTAC | + | Laxer et al. (1991) |
| LA2 | | GCTCCTCATATGCCTTATTGAG | | |
| CRY4 | SSU rRNA | gcgaatTCCTGACACAGGGAGGTAG | + | Carraway et al. (1996) |
| CRY2 | | gcgggaTCCTTGGCAAATGCTTTCG | | |
| CRIS3 | Oocyst wall | GTCCTACTGGATTCACTCTA | + | Ranucci et al. (1993) |
| CRIS6 | | GAATATGTAACACATTTATCCGC | | |
| Forward | Unknown | ATCTTCACGCAGTGCGTGGT | – | Webster et al. (1993) |
| Reverse | | CATCAGCCGGTAGATGTCGA | | |

also failed to amplify GCH1 DNA. In contrast, ribosomal primers CRY4/CRY2, primers LA1/LA2, and CRIS3/CRIS6 amplified DNA fragments in accordance with the expected sizes of 506 base pairs (bp), 449 bp and 1130 bp respectively. PCR with these three primer pairs was optimized with respect to annealing temperature and $Mg^{2+}$ concentration. Optimal annealing temperatures were 55 °C for CRY4/CRY2, 57 °C for LA1/LA2 and 61 °C for CRIS3/CRIS6. The optimal $Mg^{2+}$ concentration in the PCR mix ranged from 1.5 mM to 2.5 mM for all primer pairs without apparent differences in yield of PCR product within this range. Under these optimized conditions, the PCR yields obtained with these primer pairs were compared using serial oocyst dilutions of 1000, 100 and 10 oocysts from two *C. parvum* isolates. Primers CRY4/CRY2 and LA1/LA2 detected 10 oocysts, whereas PCR with CRIS3/CRIS6 failed to detect *C. parvum* DNA at this concentration

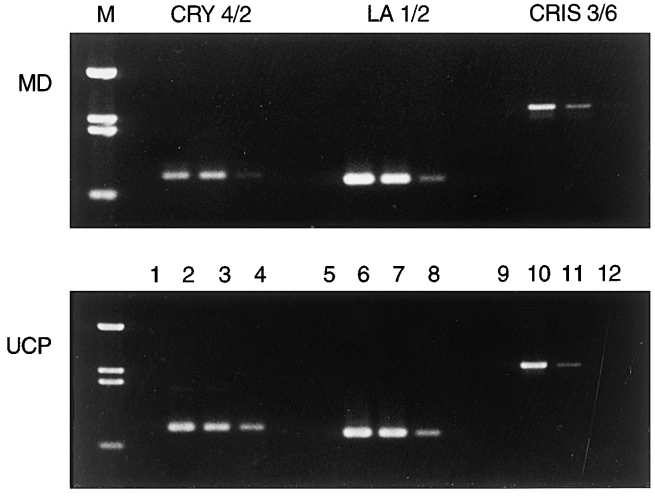


**Fig. 1** Comparison of oocyst polymerase chain reation (PCR) detection limits achieved with different primers. Tenfold serial dilutions of oocysts from *Cryptosporidium parvum* isolates MD and UCP were amplified simultaneously with three sets of PCR primers. Optimal annealing temperatures were determined experimentally: 55 °C (CRY4/2), 61 °C (LA1/2), and 57 °C (CRIS3/6). The detection limit with CRIS3/6 was around 100 oocysts (lane 11), whereas the other primers detected 10 oocysts (lanes 4, 8). No significant differences between isolates were apparent. Lanes: *2*, *6*, *10* $10^3$ oocysts; *3*, *7*, *11* $10^2$ oocysts; *4*, *8*, *12* 10 oocysts; *1*, *5*, *9* no oocysts; *M* size marker: *Bst*NI-digested pBR322 DNA (fragment sizes: 1857, 1058, 929, 383, 121 bp)

Fig. 1. On the basis of this evaluation, primers CRY4/CRY2 were retained for further optimization of *C. parvum* PCR detection.

## Oocyst disruption

The effects of different oocyst disruption procedures on the yield of PCR product were compared. Three cycles of freezing/thawing were retained as the optimal disruption method. Phenol/chloroform extraction with or without prior proteinase K digestion, either by itself, or followed by freezing/thawing did not improve PCR detection, nor did an additional two cycles (five cycles total) of freezing/thawing (not shown). Electroporation (two pulses of 2.5 kV, 129 Ω) enhanced the signal as compared to untreated oocysts, but the signal obtained was fainter than with frozen/thawed oocysts. The only method yielding results comparable to freezing/thawing was sonication; freezing/thawing was selected over sonication for subsequent experiments because of its speed and because it does not require access to specialized equipment.

## Water inhibitors

In order to investigate the presence of PCR inhibitors in surface water, lake and creek water was collected locally. Lake water collected in October and November was found to inhibit the PCR reaction strongly when present at 80% of its original concentration (Fig. 2, lanes 2, 5, 7). The inhibitory activity was stable over a period of 1 year in water samples stored at 4 °C (lane 2). In contrast, lake and creek samples collected in June and July were less or not inhibitory when added directly to PCR reactions at 80% of their original concentration (lanes 3, 4, 6). A tenfold dilution of 1995 autumn water (8% of the original concentration in the PCR reaction) resulted in complete loss of inhibition (not shown).

## Fractionation of lake water

In order to characterize further the inhibitory activity observed in lake water collected in autumn, serial filtration was performed using membrane filters of decreasing pore size. Filtrates and retentates recovered from five membrane filters were spiked with *C. parvum* DNA and assayed for PCR inhibition (Fig. 3). The

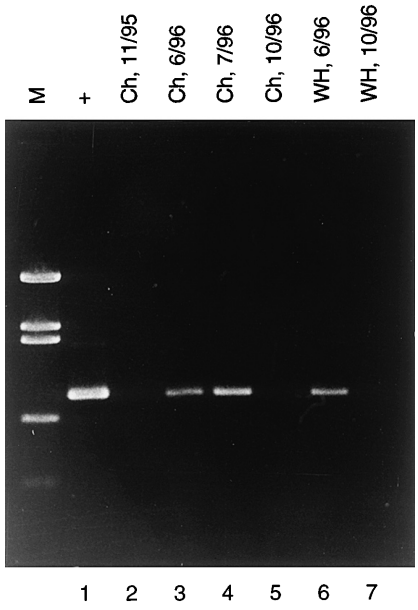


**Fig. 2** Prevalence of PCR-inhibiting activity in local surface water. Water collected from Lake Chauncy (*Ch*) in November 1995 (*2*), June 1996 (*3*), July 1996 (*4*) and October 1996 (*5*) and from an unnamed creek (*WH*) in suburban Westboro in June 1996 (*6*) October 1996 (*7*) was tested for the presence of PCR-inhibiting activity in standard PCR reactions using primers CRY4/CRY2. Inhibition of PCR was observed in autumn water samples from both water sources, as demonstrated by the absence of PCR product in lanes *2*, *5*, *7*. *1* Positive PCR control; *M* DNA size markers as in Fig. 1

result from this experiment demonstrates that the inhibitory activity in lake water resided in the soluble fraction (lanes 2, 4, 6). Partial inhibition of PCR activity in the 30-kDa filtrate (lane 8) and complete activity in the 10-kDa retentate and filtrate suggest that the inhibitory solutes were partially retained in the 30-kDa

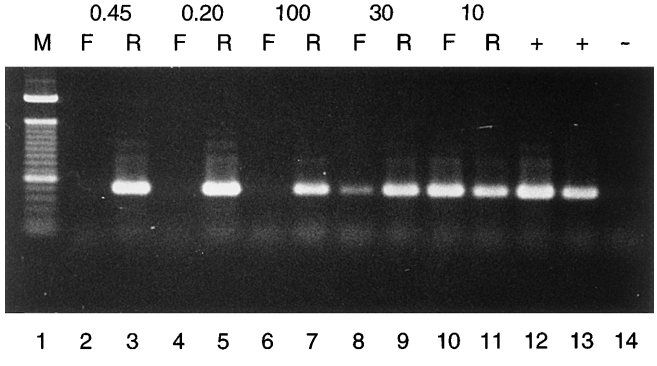


**Fig. 3** Serial filtration of PCR-inhibiting lake water. Lake water collected in autumn was filtered sequentially through membrane filters of decreasing pore size and portions of each resuspended retentate and filtrate were spiked with *C. parvum* DNA and analyzed for inhibition of PCR. Membrane pore sizes for each retentate (*R*) and filtrate (*F*) are indicated at the top. Inhibition of PCR in lanes 2, 4, 6 indicates that that PCR inhibitors are in the soluble fraction, whereas most retentates appear free from inhibiting activity. *1* 100-bp size marker; *12*, *13* positive PCR controls; *14* negative PCR control in the absence of added DNA

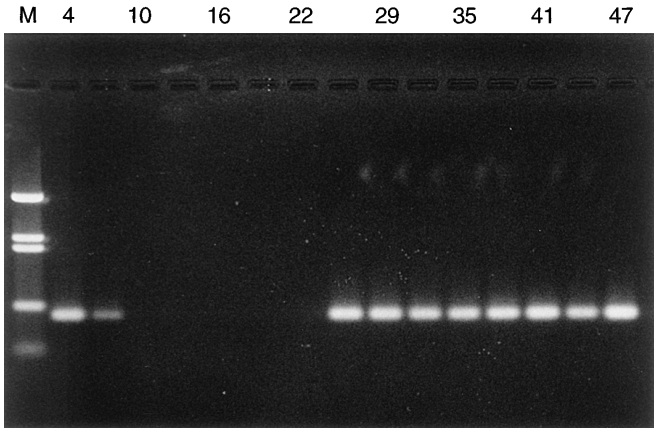


**Fig. 4** Molecular size distribution of inhibitory solutes from lake water estimated by gel filtration. Inhibitory filtrate from autumn lake water was size-fractionated by gel filtration and portions of every third fraction were assayed for the presence of inhibitors by PCR. Absence of a PCR signal in lanes 10–22, corresponding to an elution volume of 40–110 ml, is indicative of a maximal inhibitor molecular mass of 27 kDa. Fraction numbers are indicated at the top. *M* DNA size markers (see Fig. 1)

filter. For unknown reasons the inhibitory activity was not recovered in any of the retentates, although the 10-kDa filter retentate (lane 11) showed some inhibition.

A 60-kDa exclusion gel-filtration matrix was used to investigate the molecular size of inhibitory solutes further. Aliquots of 10 µl of every third fraction were assayed for PCR inhibition as above (Fig. 4). Inhibitory activity eluted in a broad peak between fractions 10 and 22, corresponding to an elution volume of approximately 40–110 ml. On the basis of elution volumes of 30 ml for ovalbumin (43 kDa) and 72 ml for ribonuclease A (13 kDa), an upper size limit of 27 kDa for the inhibitory activity was estimated by linear intrapolation. The lower limit of this activity eluted after the ribonuclease standard.

## Lake water filtrates spiked with oocysts

The filtration experiments with DNA-spiked lake water, presented in Fig. 3, suggested that PCR detection of waterborne oocysts could be significantly improved by removing inhibitory solutes. Filtration through a membrane of 0.2 µm pore size was tested to investigate whether this technique was applicable to oocyst detection using freezing/thawing as the disruption method and the optimized PCR parameters established as described above. We also wished to investigate whether increasing the ratio of waterborne particulate matter in relation to the number of oocysts would affect PCR. This question is of practical importance as routine monitoring for waterborne oocysts is performed on filtrates obtained from large volumes of water, typically several hundred liters. First, oocyst suspensions in 100 ml lake water were filtered through 0.2-µm filters, and oocyst recoveries in the retentate were determined

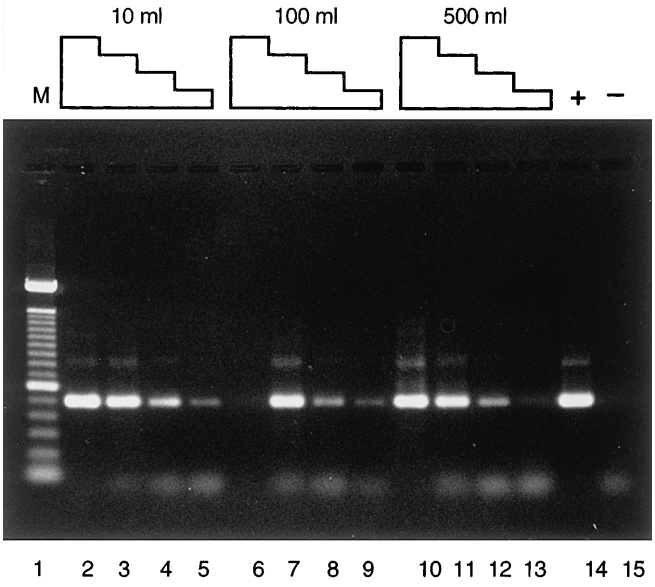


**Fig. 5** Absence of PCR inhibition in high concentrations of waterborne particulate matter. Tenfold serial dilutions of oocysts were prepared in 0.2-μm retentates obtained from 10 ml, 100 ml and 500 ml autumn lake water and assayed for PCR inhibition. Histograms indicate the number of oocysts present in the PCR reactions, ranging from $4 \times 10^4$ (lanes 2, 6, 10) to 40 (lanes 5, 9, 13). *1* 100-bp size marker; +, − positive and negative PCR controls respectively (lanes 14, 15)

by direct counting in a hemocytometer. The counts showed that more than 90% of the oocysts were consistently recovered (not shown). Retentate was then obtained from 10 ml, 100 ml, and 500 ml autumn lake water by filtration through a 0.2-μm filter, washed once with distilled water, and spiked with different concentrations of *C. parvum* oocysts. Portions of these suspensions were then frozen/thawed and assayed by 40 standard cycles of PCR (Fig. 5). The results showed that recoveries in the range of 40 oocysts were achieved regardless of the retentate concentration (lanes 5, 9, 13), indicating that no inhibition was associated with waterborne particulate matter. For unknown reasons, one reaction containing $4 \times 10^4$ oocysts (lane 6) failed to amplify a PCR product. Addition of oocysts to lake water prior to filtration did not affect PCR recoveries as compared to the above protocol, where oocysts were suspended in pre-filtered retentate. Together these observations suggest that membrane filtration efficiently removes inhibitory solutes, facilitating PCR detection of waterborne oocysts.

## Discussion

The comparison of different PCR primers specific for *C. parvum* showed that the ribosomal primers (Carraway et al. 1996) and those published by Laxer et al. (1991) gave the highest sensitivity, whereas a primer pair specific for an oocyst shell protein sequence (Ranucci et al. 1993) achieved an approximately 10-fold lower sensitivity. One set of primers (Webster et al. 1993) failed to amplify *C. parvum* DNA from three unrelated *C. parvum* isolates under different PCR conditions. The reason for primer failure is unknown, but sequence variation among isolates cannot be excluded.

Ribosomal sequences are frequently chosen for PCR amplification as the sequence is available for a large number of organisms and they are sufficiently polymorphic among species to support the development of species-specific PCR reactions. In addition, the presence of multiple copies of ribosomal genes in eukaryotic genomes can enhance the sensitivity of PCR. On the basis of the work of Zamani et al. (1996), which describes five ribosomal gene copies per haploid *C. parvum* genome, each oocyst (which contains four sporozoites) has 20 ribosomal gene copies. The copy number of the sequence targeted by LA1/LA2 and CRIS3/CRIS6 has not been reported. It would be interesting to determine to what extent the detection limits found in our experiments are affected by the copy number of the target sequence as opposed to the efficiency of the PCR reaction. This would indicate whether the design of new primers based on randomly selected sequences (Morgan et al. 1996) can lead to more sensitive PCR protocols, or whether improved PCR sensitivity will require the identification of high-copy-number sequences.

Fractionation experiments of inhibitory lake water showed that no inhibition was associated with the particulate fraction. This observation was confirmed by assaying increasing concentrations of lake water retentate for PCR inhibition. A 50-fold increase in concentration of the particulate fraction retained in a 0.2-μm filter had no apparent effect on PCR detection. This observation indicates that separation of inhibitory solutes from the particulate fraction might be sufficient to allow PCR detection of waterborne *Cryptosporidium* oocysts for routine application. However, this approach will have to be tested with the larger volumes of water required to detect small oocyst concentrations typically found in surface water.

The reason for the apparent seasonality of PCR inhibition by lake water is unknown, but we assume that temperature-dependent variability in levels of decaying organic matter are involved. A systematic comparison of solutes found in inhibitory and non-inhibitory water from the same source is in progress and will facilitate the identification of inhibitory substances. The observation that PCR inhibition was distributed over a broad range of molecular size, with an upper limit of approximately 27 kDa, is consistent with heterogeneous or polymeric substances such as humic acids (Tebbe and Vahjen 1993; Tsai and Olson 1992). We attribute the apparent discrepancy in the distribution of inhibitory activity revealed by serial filtration and gel filtration, specifically in the low-molecular-mass range, to the higher sample concentration of the gel filtration fractions. Whereas filtrates and retentates recovered from the membrane filters were tested at their original concentration, fractions

obtained by gel filtration were concentrated 15-fold. However, this does not explain the absence of inhibition in the retentates from the small-pore filters. It is possible that adherence to plastic and membranes during successive filtration steps might have reduced the concentration of inhibitors to levels no longer inhibiting PCR. This view is consistent with our observation that a 10-fold dilution of autumn lake water eliminated PCR inhibition.

In summary, using an optimized bench-scale protocol for oocyst concentration, sample preparation, and PCR, we have shown that waterborne oocysts can be reliably detected by PCR. We anticipate that the information gained from this study will facilitate field testing of this technology for monitoring waterborne *Cryptosporidium*.

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