Recovery of *Cryptosporidium* from spiked water and stool samples measured by PCR and real time PCR

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ABSTRACT: *Cryptosporidium parvum* is a common intestinal protozoan parasite infecting humans and a wide range of animals, whose diagnostics present considerable difficulties. These arise from the exceptionally robust nature of the oocyst's walls, which necessitates more stringent treatments for disruption and recovery of DNA for analysis using molecular methods. In the case of water, which is the major source of *Cryptosporidium* oocysts, investigations concern the detection of the presence of the oocysts. Their concentration in water is very low, and moreover, many substances that may have significance as inhibitors of DNA amplification, are present in environmental water and stool. We have carried out trials in order to assess the effectiveness of recovery of *C. parvum* oocysts, from spiked environmental and distilled water samples, filtrated and concentrated with the use of special laboratory equipment. Inactivation of inhibitors was carried out with use of bovine serum albumin (BSA) in PCR mixes at ten different concentrations. DNA extraction was carried out from stool samples spiked with *C. parvum* oocysts, concentrated using two methods, and unconcentrated. Nested PCR and a TaqMan nested real time PCR assay, targeting the 18S rRNA gene, was used to detect *C. parvum* DNA in spiked water and additionally in spiked stool samples. The obtained results showed that losses of *C. parvum* oocysts occur during the filtration and concentration of spiked water samples. The addition of small amounts of BSA (5–20 ng/µl) to PCR and TaqMan PCR mixes increases the sensitivity of both methods, but a high concentration of BSA (100 ng/µl and above) has an inhibiting effect on the polymerase reaction. The extraction of DNA from *C. parvum* oocysts from spiked stool samples preceded by concentration with PBS, ether and Percoll resulted in a higher copy number of the 18S rRNA gene.

Keywords: *Cryptosporidium parvum* oocysts; spiked water and stool; water filtration; stool concentration; nested PCR; TaqMan nested real time PCR; bovine serum albumin

Cryptosporidium parvum is an enteric protozoan parasite of medical and veterinary significance. Dissemination of environmentally resistant oocysts in surface water plays an important role in the epidemiology of cryptospridiosis (Smith et al. 2007). Cryptosporidiosis is an important cause of diarrhoea in young farm animals such as calves, lambs, piglets, and goat kids (Santin and Trout 2008). The infected animals suffer from mild to severe diarrhoea and respiratory disturbances that in turn adversely affect reproduction. Thus, these obligate intracellular protozoa are a potential threat to the economic development of the livestock sector (Spano and Crisanti 2000). Farmers may suffer large economic losses due to veterinary treatments, mortality and reduced growth rates (Silverlas et al. 2010). Moreover, infection of livestock with *Cryptosporidium* spp. poses a potent threat for human populations also (Peng et al. 2001). Contact with infected calves has been implicated as the cause of many small cryptosporidiosis outbreaks in veterinary students, research technicians, and children attending agricultural camps and fairs (Smith et al. 2004; Kiang et al. 2006).

Supported partially by the Ministry of Science and Higher Education of Poland (Grant No. N N404 248635).

The *Cryptosporidium* parasite is protected by an outer wall which allows it to survive outside the body for a long time. When a person or animal swallows a *Cryptosporidium* oocyst(s), the parasite exits from the wall and can cause infection. Then, more oocysts are produced and passed in the stool of the infected person or animal. In detection, the biggest difficulty is the lower concentration of oocysts in water in comparison with material collected from patients. In order to improve the monitoring of *Cryptosporidium* oocysts in water, the United States Environmental Protection Agency (USEPA) introduced the 1622 method (Anonymous 2005a) and subsequently the 1623 method (Anonymous 2005b), which consist of filtration, concentration, immunomagnetic separation (IMS), contrast staining with a fluorescent antibody and 4',6'-diamidino-2-phenylindole (DAPI), as well as microscopic detection and calculation. The 1622 method detects and calculates only *Cryptosporidium* oocysts in water samples; the 1623 method – *Cryptosporidium oocysts* and *Giardia* cysts, and introduces some improvements. However, the recovery of oocysts from environmental water samples with the use of these both methods has been reported to be very variable (Simmons et al. 2001; Kuhn and Oshima 2002; Wohlsen et al. 2004; Krometis et al. 2009; Karim et al. 2010). With regard to this fact, matrix spikes are routinely analysed to determine the influence of environmental matrices on the effectiveness of the methods for detecting target organisms (Francy et al. 2004).

The detection of the DNA of cysts and oocysts with molecular methods, in such material as surface water, sewage and wastewater or faeces, is often hampered by the presence of organic and inorganic substances that can potentially inhibit tests based on the detection of nucleic acids. For environmental samples, the efficiency of DNA extraction methods is determined by DNA recovery rate and PCR inhibitor reduction during DNA extraction. Many studies have shown that PCR inhibitors occurring in water samples suppress or reduce PCR amplification (Johnson et al. 1995; Loge et al. 2002). Environmental samples (water and soil), as well as stool samples are rich in PCR inhibitors which can be co-extracted with DNA during the DNA isolation and purification process and which can interfere with the PCR amplification (Kreader 1996; Al Soud and Radstrom 2000; Jiang et al. 2005; Plante et al. 2011; Schriewer et al. 2011).

With regard to these complications, we have carried out trials to assess the effectiveness of recovery of *C. parvum* oocysts from spiked environmental and distilled water samples. Spiked water samples were filtrated and the eluates were processed using a commercial kit for the isolation of the DNA of *Cryptosporidium* oocysts. In order to remove inhibitors, PCR reactions were carried out with the addition of bovine serum albumin (BSA) at ten different concentrations. The effectiveness of oocyst recovery and sensitivity of PCR at each BSA concentration were measured using the nested PCR signal and threshold cycle (C_T) of TaqMan nested real time PCR (targeting the 18S rRNA gene). Additionally, DNA extraction was carried out using a kit from stool samples spiked with *C. parvum* oocysts, either concentrated with PBS and ether, with PBS, ether and Percoll, or unconcentrated. The effectiveness of DNA extraction with each treatment was measured by the PCR signal of the product of the 18S rRNA gene and threshold cycle (C_T) of TaqMan nested real time PCR.

MATERIAL AND METHODS

Spiked water samples

Purified bovine-derived preparations of *Cryptosporidium parvum* oocysts (Iowa strain), were obtained from Bulk Stock Live, BTF Biomerieux, Australia. Samples containing about 5×10^3 oocysts of *C. parvum* with PBS in a volume of 100 µl were used for genomic DNA extraction. Spiked samples were prepared by adding *C. parvum* oocyst solutions containing about 5×10^3 oocysts of *C. parvum* to 10 l of distilled water and to 10 l of environmental water (Glebokie Lake). The samples were prepared with the use of a Manual Filta Max® Wash Station (Idexx Laboratories, USA) and auxiliary equipment. The ten litre water samples, seeded with oocysts, were then passed through separate compressed-foam depth filters with the use of a pump (Grundfoss, Denmark) with a flow rate of 4 l/min. Filtration and elution procedures were carried out in accordance with the manufacturer's instructions. Two hundred µl of eluate were collected for QIAamp DNA Tissue Mini Kit (Qiagen, Germany) for the extraction. Three cycles of liquid nitrogen/water bath incubation (100 °C), each for two minutes, were applied before using the kit followed by overnight incubation with proteinase K. The effectiveness of *Cryptosporidium* oocyst recovery from spiked lake and distilled water was measured by the nested

PCR signal and threshold cycle (C_T) of nested real time PCR (targeting the 18S rRNA gene). For the inactivation of PCR inhibitors, PCR reactions were carried out with the addition of bovine serum albumin (BSA) at ten different concentrations – 0, 5, 10, 15, 20, 50, 100, 200, 300 and 400 ng/µl.

Spiked stool samples

Parasite-free stool samples in volumes of 200 µl (in triplicates) were used for spiking with 5×10^3 oocysts of *C. parvum*. DNA from the first sample was isolated without concentration, from the second one – using the PBS and ether method, and from the third one – using PBS, ether and Percoll (Sigma, Germany) method (both methods are described in Waldman et al. (1986).

The QIAamp DNA Stool Mini Kit (Qiagen) was used for DNA extraction according to the manufacturer's instructions, preceded by three liquid nitrogen/water bath (100 °C) incubations (each for two minutes). The DNA Stool Mini Kit contains InhibitEX Tablets for adsorption and for the removal of stool compounds that may degrade DNA or inhibit PCR. Their effectiveness was evaluated with PCR and real time PCR after extraction with each treatment.

Nested PCR amplification

A highly polymorphic region of the 18S rRNA gene was amplified in the nested PCR protocol. The method involves the amplification of an approximately 1325 bp long primary product followed by a secondary amplification of an internal fragment with a length of approximately 840 bp. For the first PCR step, a PCR product was amplified using the primers CX1F 5'-TTCTAGAGCTAATACATGCG-3' and CX1R2 5'-CCCTAATCCTTCGAAACAGGA-3'. For the second PCR step, a PCR product was amplified using 1 µl of the primary PCR product and the primers CX2F 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and CX2R 5'-AAGGAGTAAGGAACAACCTCCA-3' (Xiao et al. 2001). Each PCR mixture (total volume 10 µl) contained 5pM of each primer (Genomed, Poland), 0.3mM of each deoxynucleotide triphosphate (Novazym, Poland), 1 µl of 10 × PCR buffer, 30mM $MgCl_2$, 0.5 IU of *Taq* polymerase (Sigma, Germany), and 1 µl of DNA template. The reactions were performed in a DNA thermal cycler (Biometra, Germany and MJ Research, USA). Thermal-time profiles in the first and second PCR were the same as described by Xiao et al. (2001). Negative control reaction mixtures contained sterile distilled water in place of DNA template. PCR products were visualized in 1.5% agarose gels stained with ethidium bromide. All analyses were carried out in two replicates.

TaqMan nested real time PCR

A region of the small subunit of the rRNA gene of *C. parvum* was utilised as a target sequence for nested real time PCR. The primers CPrI and CPrII amplifying a 676 bp fragment (Bialek et al. 2002; Minarovicova et al. 2009), were used as outer primers. The inner primers, CPrF3 and CPrR3, amplifying a 118 bp fragment and a TaqMan probe, CPrP3-1, were designed by Minarovicova et al. (2009). Each PCR mixture (total volume 10 µl) contained 8pM of each outer primer and the probe (Genomed, Poland), 0.9nM of each dNTP (Novazym, Poland), 1 µl of 10 × PCR buffer, 25mM $MgCl_2$, 1.5 IU Taq DNA Polymerase (Sigma, USA) and 2 µl of DNA template. Reactions were performed in Rotor Gene 6000 (Corbett Research, Sydney, Australia). All oligonuceotide sequences and thermal profiles were as described previously (Minarovicova et al. 2009). The threshold cycle (C_T) was calculated for individual samples using the software of the cycler with the manual threshold set at a fluorescence value of 10^{-2}. Appropriate negative controls were included in each PCR run. All analyses were carried out in two replicates.

RESULTS

The effectiveness of recovery of *C. parvum* oocysts from spiked distilled and lake water, measured by the intensity of the nested PCR signal and C_T TaqMan nested real time PCR values, are shown Table 1, Figures 1 to 3 (paths 1–4) . The intensity of signal obtained with nested PCR was the strongest for *C. parvum* oocysts in PBS, and the weakest for spiked and filtrated lake water. Real time nested PCR results, measured using the values of the threshold cycle (C_T), confirmed the results of nested PCR.

The addition of bovine serum albumin to the nested PCR mix, for samples isolated from spiked

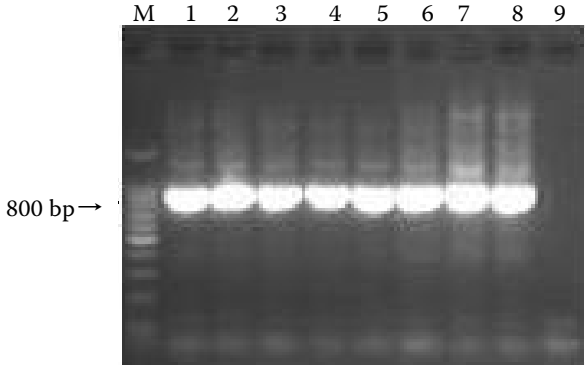

Figure 1. Products of amplification of a fragment of the 18S SSU rRNA gene of *Cryptosporidium* sp. obtained from DNA isolated from *C. parvum* oocysts in PBS without concentration (paths 1–8); M = molecular weight marker, path 9 = negative control

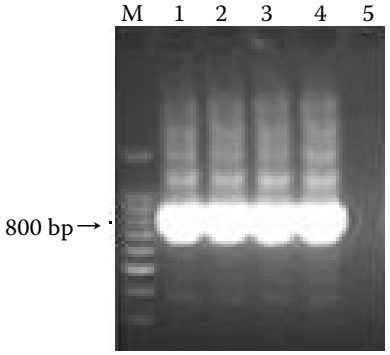

Figure 2. Products of amplification of a fragment of the 18S SSU rRNA gene of *Cryptosporidium* sp. obtained from DNA isolated from spiked distilled water (paths 1–4), path 5 = negative control, M = molecular weight marker

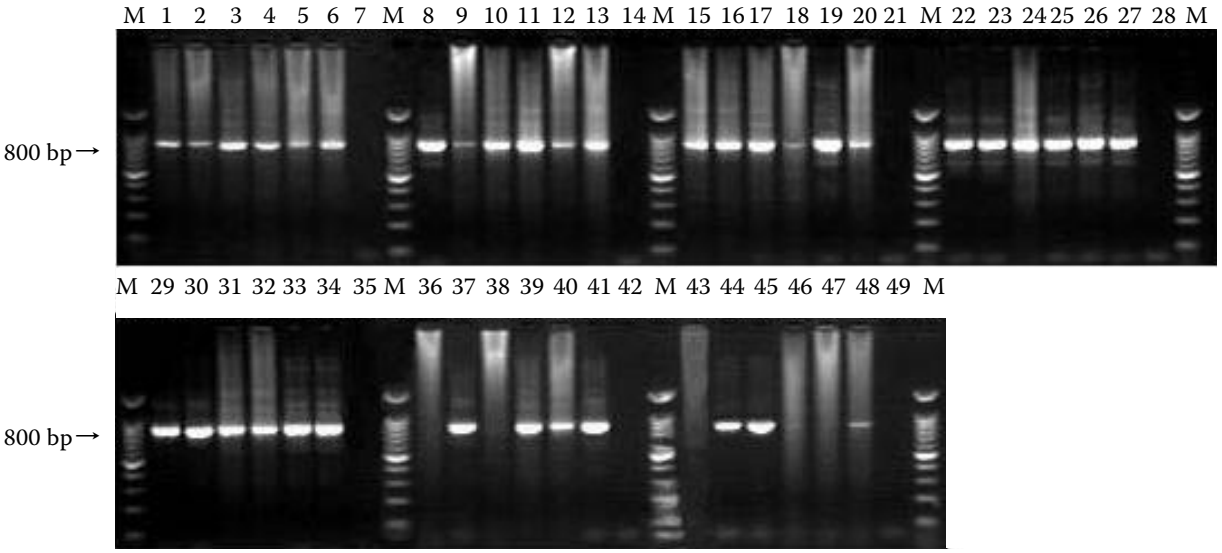

Figure 3. Products of amplification of a fragment of the 18S SSU rRNA gene of *Cryptosporidium* sp. obtained from DNA isolated from spiked lake water using the following mixes: without BSA (paths 1–4), with BSA at a concentration of 5 ng/µl (paths 8–11), with BSA at a concentration of 10 ng/µl (paths 15–18), with BSA at a concentration of 15 ng/µl (paths 22–25), with BSA at a concentration of 20 ng/µl (paths 29–32), with BSA at a concentration of 50 ng/µl (paths 36–39), with BSA at a concentration of 100 ng/µl (paths 43–46); paths 5, 6, 12, 13, 19, 20, 26, 27, 33, 34, 40, 41, 47, 48 = positive control, paths 7, 14, 21, 28, 35, 42 and 49 = negative control, M = molecular weight marker

lake water, caused the largest increase in reaction sensitivity at concentrations of 15 ng/µl and 20 ng/µl (Table 2, Figure 3). For real time nested PCR, the greatest increase in reaction sensitivity was elicited by BSA at concentrations of 5 ng/µl and 10 ng/µl (Table 2).

The intensity of the signal obtained using the nested PCR method was the same for all three procedures preceding the isolation of DNA from *Crytosporidium parvum* oocysts from spiked stool samples (Table 3, Figure 4). C_T values obtained in the TaqMan nested real time PCR reaction were highest after the separation of oocysts with the use of PBS, ether and Percoll, and the lowest after DNA extraction of unconcentrated oocysts (Table 3).

Table 1. Effectiveness of recovery of *C. parvum* oocysts from spiked distilled and lake water measured by nested PCR and TaqMan nested real time PCR

	Results of nested PCR	Results of TaqMan nested real time PCR (threshold cycle – C_T)
C. parvum oocysts with PBS	+ + + +	4.32 ± 0.34
spiked and filtrated distilled water	+ + +	8.25 ± 0.24
spiked and filtrated lake water	+ +	11.38 ± 0.58

++++ = very strong intensity of the DNA band, +++ = strong intensity of the DNA band, ++ = medium intensity of the DNA band

Table 2. Sensitivity of nested PCR and TaqMan nested real time PCR of spiked lake water samples with the addition of BSA t ten different concentrations, measured by the signal of nested PCR and threshold cycle (C_T) of real time PCR

Concentration of BSA (ng/µl)	Results of nested PCR	Results of TaqMan nested real time PCR (threshold cycle – C_T)
0	+ +	18.3 ± 0.43
5	+ + +	4.3 ± 0.85
10	+ + +	6.8 ± 0.45
15	+ + + +	14.2 ± 0.43
20	+ + + +	14.3 ± 0.23
50	+ +	14.8 ± 0.48
100	+	24.5 ± 0.8
200	–	38.7 ± 0.15
300	–	–
400	–	–

++++ = very strong intensity of the DNA band, +++ = strong intensity of the DNA band, ++ = medium intensity of the DNA band, + = weak intensity of the DNA band, – = no DNA band visualised on ethidium bromide-stained agarose gel

Table 3. Effectiveness of DNA extraction from stool samples spiked with *C. parvum* oocysts unconcentrated, concentrated with PBS and ether or with PBS, ether and Percoll, measured by the signal of nested PCR and threshold cycle (C_T) of real time PCR

Samples	St kit	
	results of nested PCR	results of TaqMan nested real time PCR (threshold cycle – C_T)
Unconcentrated	+++	10.46 ± 0.84
PBS + ether	+++	8.38 ± 0.42
PBS + ether + Percoll	+++	6.73 ± 0.35

+++ = strong intensity of the DNA band

DISCUSSION

Although only ten years have passed since the United States Environmental Protection Agency (USEPA) introduced the 1622 and 1623 methods (Anonymous, 2005a,b), these are in need of improvement (Hu et al. 2004; Skotarczak 2009, 2010; Adamska et al. 2010). In both methods, the filtration of water samples of 10–50 l volume is carried out with the use of several kinds of filters in accordance with the producer's recommendations. The next step is concentration of the eluate washed out from the filter by suction with a vacuum pump. Both methods recommend immunomagnetic separation (IMS) as a next step. IMS is used to separate cysts and oocysts from foreign material (Shaw et al.

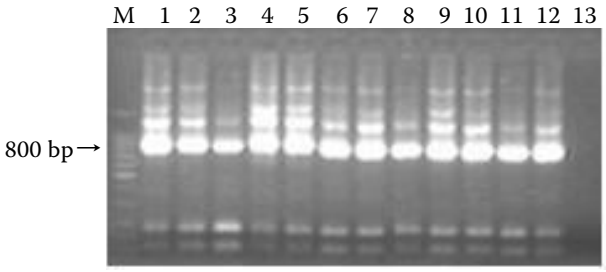

Figure 4. Products of amplification of a fragment of the 18S SSU rRNA gene of *Cryptosporidium* sp. obtained from DNA isolated from spiked stool samples; paths 1–4 = amplicons obtained from DNA isolated from unconcentrated oocysts, paths 5–8 = amplicons obtained from DNA isolated from oocysts concentrated with PBS and ether, paths 9–12 = amplicons obtained from DNA isolated oocysts concentrated with PBS, ether and Percoll, path 13 = negative control, M = molecular weight marker

2008); however, this method becomes impractical for organisms that have no IMS procedures. The use of IMS is also expensive, and this limits the use of samples mostly to single-organism detection (Jiang et al. 2005). However, IMS is not only expensive; its performance is affected by the type of commercial kit used, pH, and dissociation procedures (Ware et al. 2003). Earlier studies have shown various degrees of recovery efficiency for *Cryptosporidium* (Hsu et al. 2001; Simmons et al. 2001; Wohlsen et al. 2004). Most noted a decrease in the recovery level of *C. parvum* oocysts after filtration (Feng et al. 2003; Hu et al. 2004).

Our preliminary studies allowed us to determine that the extraction of DNA from *C. parvum* oocysts is most effective when it is preceded with cycles of liquid nitrogen/water baths incubation and with the use of lysate buffer and overnight proteinase K digestion (Adamska et al. 2011). We have applied the same procedures to assess the extraction of oocyst DNA recovered from distilled water, environmental samples and spiked stool samples. To this end, we have attempted to adapt and develop PCR and TaqMan nested real time PCR assays for the direct detection of oocysts DNA, because it is very significant for the detection of pathogens in environmental samples.

The nested PCR signal of products of the 18S rRNA gene for oocysts recovered from lake and distilled water was less intense than for isolates coming directly from *C. parvum* oocysts in PBS. Moreover, the C_T values for DNA isolates coming directly from *C. parvum* oocysts in PBS were at least two times lower than for isolates coming from spiked water, which testifies to higher original number of DNA copies in this isolate and to losses of oocysts during the filtration of spiked water samples. On the other hand, the decline in recovery efficiency measured by nested PCR and TaqMan nested real time PCR was relatively low (Table 1), probably because of the use of a great number of oocysts. However, *Cryptosporidium* oocysts are not numerous in environmental samples and even small losses may have a significant influence on their detection.

DNA extraction from spiked stool samples was carried out with *C. parvum* oocysts that were unconcentrated, concentrated with PBS + ether or with PBS + ether + Percoll and then extracted using an agent kit. The effectiveness of DNA extraction with each treatment was measured by the nested PCR signal and threshold cycle (C_T) of nested real time PCR. The nested PCR signals of the products of the 18S rRNA gene for oocysts recovered from stool, isolated with all pre-isolation treatments, were very similar (Table 3, Figure 4). Based on the assumption that there exists an inverse relationship between the number of DNA copies originally present in the isolate and the C_T value, the effectiveness of DNA extraction is highest when preceded by concentration with PBS + ether + Percoll (Table 3). However, differences between C_T values in nested real time PCR, for isolates treated using various methods, were not large. The DNA Stool Mini Kit used for DNA extraction contains "InhibitEX Tablets" that, according to the producer adsorb and remove all typical stool compounds that can degrade DNA or inhibit molecular reactions. These tablets seem to be effective in removing inhibitors of downstream enzymatic reactions.

Additional procedures have been developed and are still being improved, for the removal or reduction of the effects of PCR inhibitors such as tannic, humic and fulvic acids which are abundant in natural waters (Kreader 1996; Al Soud and Radstrom 2000; Guy et al. 2003; Skotarczak 2009; Plante et al. 2011; Schriewer et al. 2011). In our studies, the differences between the nested PCR signals and C_T values obtained for the DNA of oocysts recovered from lake and from distilled water implicate the presence of inhibitors in environmental water. The reduction or removal of PCR inhibitors is an essential component in the molecular detection of microorganisms in environmental samples, because inhibitors may interfere with the cell lysis

step, inactivate the thermostable DNA polymerase and/or interfere with nucleic acids (Al Soud and Radstrom 2000; Plante et al. 2011; Schriewer et al. 2011). The reduction of PCR inhibitors before, during, or after DNA extraction has become an important step in the molecular diagnosis of protozoan pathogens in water and other environmental samples (Jiang et al. 2005).

Many chemicals are used to deactivate PCR inhibitors, e.g., acetamide, betaine, dextran, glycerol, formamide, pectinase, polyvinylpyrrolidene, aqueous solutions of non-fat dry milk or bovine serum albumin (BSA) (Al Soud and Radstrom 2000; Plante et al. 2011). Earlier studies revealed that BSA is one of the most efficient facilitators of conventional PCR (Al Soud and Radstrom 2000; Skotarczak 2009; Plante et al. 2011; Schriewer et al. 2011). Bovine serum albumin binds to many substances and prevents their binding to Taq DNA polymerase, target DNA and magnesium ions (Kreader 1996; Plante et al. 2011). BSA has been widely used to reduce inhibitory effects in PCR assays of protozoans (Kreader 1996; Al Soud and Radstrom 2000; Jiang et al. 2005; Schriewer et al. 2011); however, studies reporting the optimisation of BSA concentrations for real time PCR are rare (Plante et al. 2011; Schriewer et al. 2011). A wide range of BSA concentrations have been utilised (from 20 ng/µl to 400 ng/µl) (Kreader 1996; Al Soud and Radstrom 2000; Jiang et al. 2005; Schriewer et al. 2011). According to Kreader (1996), the optimum concentration of BSA for the relief of humic acid inhibition ranges between 200 and 400 ng/µl. In the study of Jiang et al. (2005), four different concentrations were used: 50, 200, 400 and 600 ng/µl. The effect of PCR inhibitors was reduced significantly with 400 ng of BSA per 1 µl. Schriewer et al. (2011) used BSA concentrations in the range from 50 to 200 ng/µl in real time PCR mixes and their studies indicate that there is no variance elicited by differing amounts of BSA. These results indicate that high concentrations of BSA do not increase the sensitivity of real time PCR. Guy et al. (2003) reported that DNA extracts obtained from samples of environmental water were too abundant in inhibitors for analysis using molecular methods. However, the addition of BSA at a final concentration of 20 ng/µl to the real time PCR mixture removed the inhibitory effect.

Real time PCR is more sensitive than conventional PCR; however, excessively high BSA concentrations, equal to or greater than 100 ng/µl, may have negative effects on the detection limit (Schriewer et al. 2011). The results obtained in our studies confirm that high concentrations of BSA (100 ng/µl and above) have an inhibiting effect on the polymerase reaction (Table 2). The addition of bovine serum (BSA) at concentration ofs 15–20 ng/µl to the nested PCR mix and 5–10 ng/µl to the TaqMan PCR mix, for samples isolated from spiked lake water, caused an increase in reaction sensitivity. Using reaction mixes without the addition of BSA resulted in quite strong PCR signals, but in the case of TaqMan real time PCR, the C_T values were relatively high (18.3) (Table 2). The optimum range of applied BSA may vary depending on the amount of inhibitors and the matrix composition (Schriewer et al. 2011), and so the type of environmental sample (e.g., lake, river pound or tap water) and of detected organism may influence the optimal BSA concentration.

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Received: 2011–07–06
Accepted after corrections: 2012–05–24

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