A fast and sensitive nucleic acid extraction method for the detection of Cryptosporidium by PCR in environmental water samples

J. Dellundé, S. Pina, J. Jofre and F. Lucena

Departament of Microbiology, University of Barcelona, Spain (E-mail: dellunde@porthos.bio.ub.es; spina@porthos.bio.ub.es; joan@porthos.bio.ub.es; lucena@porthos.bio.ub.es)

Abstract A new protocol based on a combination of an excystation process followed by a nucleic acid extraction that combines the lysing and nuclease-inactivating properties of the chaotropic agent guanidinium thiocyanate and the nucleic acid-binding properties of silica particles is described for the extraction and purification of nucleic acids from Cryptosporidium. The application of nested and/or semi-nested PCR using different external and internal primers to DNA extracted by this method from seeded and naturally occurring Cryptosporidium oocysts concentrated and purified from environmental samples detects numbers of oocysts ranging from 20 to 50. The method is feasible, detects mostly excystable oocysts and no problems of inhibition of PCR were observed when applied to environmental samples.

Keywords Cryptosporidium; environmental; excystation; PCR

Introduction

In the last decade, genetic methods, which are based on detection of specific nucleic acid, mostly those based in amplification techniques have been developed for the detection of Cryptosporidium (Johnson et al., 1995; Laxer et al., 1991; Rochelle et al., 1997; Wagner and Kimming, 1995). Though numerous protocols for nucleic acid extraction had been described, efficient nucleic acid extraction from Cryptosporidium oocysts is a difficult task, making difficult the detection and characterisation of Cryptosporidium oocysts present in environmental water samples and the determination of their viability by targeting the mRNA of HSP 70 heat-shock protein (Rochelle et al., 1997; Stinear et al., 1996). Some of these methods such as sonication, freeze thawing, and glass bead disruption are frequently employed yet requiring additional enzymatic steps for giving a good extraction nucleic acid efficiency (da Silva et al., 1999). All together the extraction of nucleic acids from Cryptosporidium oocysts is a cumbersome and long lasting process.

A new method is proposed in the present report. It is based on a combination of an excystation process (Campbell et al., 1992) followed by a nucleic acid extraction that combines the lysing and nuclease-inactivating properties of the chaotropic agent guanidinium thiocyanate together with the nucleic acid-binding properties of silica particles (Boom et al., 1990). Different experiments to assess the applicability of the method to the extraction of DNA for DNA amplification as well to the extraction of mRNA for RT-PCR amplification are presented.

Materials and methods

Nucleic acid extraction

Excystation. 200 µl of bile solution (1% bovine bile in Hanks minimal essential medium) and 50 µl of sodium hydrogen carbonate solution (0.44% sodium hydrogen carbonate in RO water) were added to 100 µl of purified oocysts previously pre-treated with Hank’s Balanced Salt Solution at pH 2.75 for 1 hour at 37°C. The mixture was then incubated during 4 hours at 37°C (Campbell et al., 1992).
Nucleic acid extraction and purification. First, the pre-assembled reaction vessel containing buffer lysis (GuSCN 10M, EDTA and Triton x100) and silica particles was vortexed to homogeneity. A 50 µl sample of excysted oocysts was then added and the mixture was immediately vortexed (approximately 5 s). After 10 minutes at room temperature, the vessel was vortexed again (5 s) and centrifuged (15 s) in an Eppendorf microfuge (fixed angle, 14,000 × g). The supernatant was disposed of by suction. The silica-nucleic acid pellet was subsequently washed twice with washing buffer, twice with ethanol 70% (vol/vol) and once with acetone. After disposal of the acetone, the vessels are dried at 56°C with open lids in an Eppendorf heat block for 10 min. Elution buffer (TE buffer with or without RNase inhibitor) was added (50 µl), the vessel was closed, then vortexed briefly and finally incubated for 10 minutes at 56°C with open lids. The vessel was closed, vortexed briefly again and centrifuged for 2 min at 12,000 × g and the supernatant containing DNA and RNA was used for both PCR and RT-PCR amplifications.

A set of parallel experiments was performed with oocysts not previously submitted to the excystation steep.

PCR conditions and gel electrophoresis

10 µl of the nucleic acid solutions were added to microtubes containing 40 µl portions of PCR mixture, that contained 2 units of AmpliTaq DNA polymerase (Perkin-Elmer, Roche), 10 mmol l⁻¹ Tris-HCl (pH 8.3), 50 mmol l⁻¹ KCl, 1.5 mmol l⁻¹ MgCl₂, 250 µmol l⁻¹ of each dNTP and 0.5 µmol l⁻¹ of each primer (Table 1). The preparations in the microtubes were initially denatured at 95°C for 3 minutes and then subjected to 30 cycles of denaturation at 94°C for 1 minute, annealing at 45°C or 50°C for 1 minute depending on the set of primers used, extension at 72°C for 1 minute and finally one cycle of final extension at 72°C for 5 minutes in a GeneAmp 2400 PCR thermal-cycler (Perkin-Elmer, Applied Biosystems).

A 1 µl portion of the first step PCR products was added to 49 µl of the PCR mixture containing the set of internal primers (see Table 1) for the second-step PCR (semi nested or nested PCR) which was performed with an initial denaturation step at 95°C for 3 minutes and then subjected to 30 cycles of denaturation at 94°C for 1 minute, annealing at 45°C or 50°C for 1 minute depending on the set of primers used, extension at 72°C 1 minute and finally one cycle of final extension at 72°C for 5 minutes. PCR-amplified fragments (10 µl each) were separated in 2 or 3% agarose gels.

RT-PCR conditions and gel electrophoresis

5 µl aliquots of the nucleic acid solutions were mixed with 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01 M DTT, 250 µM of each 2’-deoxynucleoside 5’-triphosphate (dNTP) and 0.5 µM of oligo (dT)$_{25}$ primer to a total volume of 10 µl. The mixture was incubated at 95°C for 5 min before the addition of 10 U of RNase inhibitor and 50 U of reverse transcriptase Moloney murine leukaemia virus (MMLV) (Perkin-Elmer Roche, Inc). After 30 min at 42°C, the reaction was again heated for 5 min at 95°C.

For a typical one-step reaction, 10 µl aliquots of the cDNA solution were used. Amplification was carried out in a 50 µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 250 µM of each dNTP, 0.5 µM of each primer (see table 1) and 2U of AmpliTaq DNA polymerase (Perkin-Elmer, Roche). The amplification reaction was performed in a GeneAmp 2400 PCR thermal-cycler (Perkin-Elmer, Applied Biosystems). The first cycle of denaturation was carried out for 3 min at 95°C, followed by 30 cycles of denaturation at 94°C for 1min, annealing at 50°C for 1 min, extension at 72°C for 1 min, and 1 cycle of final extension at 72°C for 5 min. A 1 µl portion of the first step PCR products was added to 49 µl of the PCR mixture that containing the set of internal
The second-step (semi nested or nested PCR) was performed with an initial denaturation step at 95°C for 3 minutes and then subjected to 30 cycles of denaturation at 94°C for 1 minute, annealing at 50°C 1 minute, extension at 72°C for 1 minute and finally one cycle of final extension at 72°C for 5 minutes. PCR-amplified fragments (10 µl each) were separated in 2 or 3% agarose gels.

**Sensitivity of the detection method**

*Dilution of nucleic acid solutions after the extraction procedure.* Serial 10 fold dilution of nucleic acid extracted and purified from aliquots of stock suspension oocysts previously titrated by laser scanning cytometry and epifluorescence microscopy were performed in elution buffer. The sensitivity of the method was determined by amplifying the nucleic acids following the two-step PCR and RT-PCR procedure described above.

*Dilution of oocysts suspensions previous to the extraction procedure.* Nucleic acids were extracted and purified from two fold dilutions in phosphate buffer saline of oocysts suspensions previously titrated by laser scanning cytometry. Each dilution was divided into two equivalent aliquots. The nucleic acids were then extracted from one of the aliquots and the oocysts present in the second aliquot were enumerated by laser scanning cytometry. The sensitivity of the method was determined by amplifying the nucleic acids following the two-step PCR and RT-PCR procedures described above.

*Dilution of nucleic acids after extraction from the concentrate of 1 litre of river water seeded with oocysts.* Serial 10 fold dilutions of nucleic acid purified from the concentrate of one litre of river water seeded previously with a known number of oocysts were performed in elution buffer. The sensitivity of the method was determined by amplifying the nucleic acids following the two-step PCR procedure described above. The concentration of oocysts was performed using the protocol described by Falk et al., 1998 and then purified by immunomagnetic separation (IMS).

### Table 1

Sets of primers used in this work. The primer NST1 was designed in this study using the sequences deposited in European Molecular Biology Library and GenBank and the software BLASTN v.2.0.8, FAST A and CLUSTALW 1.74

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Primer</th>
<th>Sequence</th>
<th>Tm</th>
<th>Amplified fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cryptosporidium</em> spp</td>
<td>CPHSP2423*</td>
<td>5' -AAATGGTGAGCAATCCTCTG-3'</td>
<td>58°C</td>
<td>360 bp</td>
</tr>
<tr>
<td></td>
<td>CPHSP2764*</td>
<td>5' -CTTGCTGCTCTTTACCAGTAC-3'</td>
<td>60°C</td>
<td></td>
</tr>
<tr>
<td><em>C. parvum</em></td>
<td>CHSP1*</td>
<td>5' -AGCAATCCTGTAGGATCTTCTTCT-3'</td>
<td>66°C</td>
<td>590 bp</td>
</tr>
<tr>
<td></td>
<td>CHSP4*</td>
<td>5' -AAGAGCATCCTTTGATCTTCT-3'</td>
<td>56°C</td>
<td></td>
</tr>
<tr>
<td><em>C. parvum</em></td>
<td>CPR1d#</td>
<td>5' -GCCGCACCTGGATATACACTTCTTCTTCTC-3'</td>
<td>66°C</td>
<td>358 bp</td>
</tr>
<tr>
<td></td>
<td>CPR1r#</td>
<td>5' -TCCCCCTCTCTCTAAGGAGAAG-3'</td>
<td>64°C</td>
<td></td>
</tr>
<tr>
<td><strong>SEMI / NESTED PCR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cryptosporidium</em> spp</td>
<td>NST1</td>
<td>5' -GTTCGCTAAATTACACTCCTG-3'</td>
<td>58°C</td>
<td>321 bp</td>
</tr>
<tr>
<td></td>
<td>CPHSP2764*</td>
<td>5' -CTTGTGCTCTTACCAGTAC-3'</td>
<td>60°C</td>
<td></td>
</tr>
<tr>
<td><em>C. parvum</em></td>
<td>NST1</td>
<td>5' -GTTCGCTAAATTACACTCCTG-3'</td>
<td>58°C</td>
<td>551 bp</td>
</tr>
<tr>
<td></td>
<td>CHSP4*</td>
<td>5' -AAGAGCATCCTTTGATCTTCT-3'</td>
<td>56°C</td>
<td></td>
</tr>
<tr>
<td><em>C. parvum</em></td>
<td>NCPR1d#</td>
<td>5' -GATCCCAATGCGAGAAT-3'</td>
<td>51°C</td>
<td>310 bp</td>
</tr>
<tr>
<td></td>
<td>NCPR1r#</td>
<td>5' -CAAACGTATTGAAAGAGC-3'</td>
<td>48°C</td>
<td></td>
</tr>
</tbody>
</table>

# Chung et al., 1998
* Kauckner and Stinear, 1996
Natural water samples
Oocysts from 9 sewage and 12 river water samples were concentrated by filtration as indicated above and the concentrated oocysts reconcentrated and purified by immunomagnetic separation (IMS): the final concentrates were divided into two aliquots. The nucleic acids were then extracted from one of the aliquots and amplified following the two-step PCR procedures described above. The oocysts present in the second aliquot were enumerated by LSC.

Laser scanning cytometry
Laser scanning cytometry experiments were carried out using a laser scanning cytometer system consisting of a 5 mW argon-ion laser (488 nm) and helium-neon laser (633 nm), 4 photomultiplier (PTM) assemblies with interchangeable filter blocks, forward scatter assembly and Olympus BX50 microscope. Forward scatter area, green fluorescence integral and green fluorescence maximum pixel were collected (logarithmic scale). Cryptosporidium stained with fluoresceine conjugated monoclonal antibody (Crypt-a-Glo, Waterbone) was detected by combining gates on both the forward scatter area FITC integral and FITC integral – FITC maximum pixel dot plots. Samples were analysed inside a chamber with 5 µl capacity and a slide area of 2.5 × 20 mm.

Results and discussion
Sensitivity of the semi nested or nested PCR
Tests were performed to determine the detection limit of the second PCR step by serial 10 fold dilutions of DNA solutions extracted and purified as described above. Gels in Figure 1 indicate detection limits lying between 0.3 and 3 oocysts (equivalent to 1.2 to 12 genomes). Similar results were obtained in repeated experiments for the different sets of external and internal primers tested (seminested PCR for gene hsp70 – primers for nonspecific and specific for the detection of C. parvum, nested PCR for gene cpr1 for C. parvum).

When the amplification was applied to DNA extracted without the excystation step, the detection limit was around 1,000 oocysts. This indicates that the complete method described herein mainly applies for excystable oocysts.

To adjust the real detection limit of the PCR procedure using the nucleic acid extraction method defined in this work we assess the detection limit using a known number of pure oocysts enumerated in each sample by LSC. A detection limit between 10 to 50 oocysts per PCR assay was achieved. Table 2 summarizes one of the experiments. Similar sensitivities had been reported by others authors (Chung et al., 1998; Jonhson et al., 1995; Rochelle et al., 1997; Sluter et al., 1997).

When the detection limit of PCR assay was determined with serial 10 fold dilutions of the nucleic acids extracted from purified oocysts previously seeded into 1 litre of river water, concentrated by filtration technique and purified by immunomagnetic separation (IMS), the detection limit decreased to between 1.2 and 12 oocysts (equivalent to 5 and 50 genomes) (Figure 2), probably due to losses during concentration. This lower detection limit was observed for all sets of primers used. Interestingly enough it does not seem to be substances inhibiting the PCR reaction. This contrasts with the high inhibition reported by other authors in lake water (Chung et al., 1998; Jonhson et al., 1995; Sluter et al., 1997). This difference may be due to the fact that the potential inhibitory substances present in the concentrate of river water such as humic acids, nucleases or some polysaccharides would be eliminated first by the IMS purification process and second by the nuclease-inactivating properties of the chaotropic agent guanidinium thiocyanate.
Detection of naturally occurring *Cryptosporidium* ssp and *Cryptosporidium parvum* in polluted waters

Results are shown in Table 3. Samples with the number of oocysts higher than 30 gave positive PCR amplification. These results are in concordance with the detection limit determined in the experiments reported above (between 10 to 50 oocysts). It is necessary to emphasize that 5 to 15% of oocysts present in the concentrates of natural samples were empty, data very important for determining the lower oocysts concentration detected by PCR.

The presence of *Cryptosporidium parvum* oocysts was also analyzed in the natural water sample analyzed. All the 9 sewage water samples analyzed were positive for *Cryptosporidium parvum* oocysts. On the contrary only 2 of the 3 river water samples positive for *Cryptosporidium* were positive for *C. parvum*.

Interesting to mention is that, in contrast with other methods, no problems of inhibitors of the PCR were observed either with sewage samples or with river water samples.

Table 2 Determination of sensitivity level of PCR in oocysts seeded in river water. Oocysts were concentrated prior to enumeration by LSC and extraction of nucleic acids. These results correspond to an experiment performed in triplicate. Detection of oocysts after 30 amplification cycles using external CHSP1 and CHSP4 for *Cryptosporidium parvum* (PCR) followed by 30 amplification cycles using internal primers NST1 and CHSP4 for *Cryptosporidium parvum* (nested PCR).

<table>
<thead>
<tr>
<th>Oocysts enumerated by LSC*</th>
<th>1050</th>
<th>439</th>
<th>225</th>
<th>106</th>
<th>43</th>
<th>11</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Nested PCR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Laser Scanning Cytometry

Detection of naturally occurring *Cryptosporidium* ssp and *Cryptosporidium parvum* in polluted waters

Results are shown in Table 3. Samples with the number of oocysts higher than 30 gave positive PCR amplification. These results are in concordance with the detection limit determined in the experiments reported above (between 10 to 50 oocysts). It is necessary to emphasize that 5 to 15% of oocysts present in the concentrates of natural samples were empty, data very important for determining the lower oocysts concentration detected by PCR.

The presence of *Cryptosporidium parvum* oocysts was also analyzed in the natural water sample analyzed. All the 9 sewage water samples analyzed were positive for *Cryptosporidium parvum* oocysts. On the contrary only 2 of the 3 river water samples positive for *Cryptosporidium* were positive for *C. parvum*.

Interesting to mention is that, in contrast with other methods, no problems of inhibitors of the PCR were observed either with sewage samples or with river water samples.
Determination of oocysts viability by RT-PCR of the mRNA of the hsp70 gene

Some protocols for the determination of oocysts viability by RT-PCR based on the detection of mRNA of HSP 70 heat shock protein have been described (Kaucner, 1998; Stinear et al., 1996).

The method proposed in this work permit us to include the possibility to introduce an additional heat shock step (44°C, 30 min) for targeting the mRNA. The first results with serial dilutions of extracted and purified mRNA showed a sensitivity ranging from 10 to 100 viable oocysts. These results indicate that the method should be improved before applying it to environmental samples.

In conclusion we have developed a feasible method for nucleic acid extraction that permits the repeatable detection by nucleic acid amplification in environmental samples of numbers of excystable oocysts, whatever the meaning of excystability, ranging from 20 to 50 oocysts.

Acknowledgements

This study was partially supported by FEDER (2FD1997-1104), and by the International Co-operation Developing Countries (INCO-DC) EU project ERB 3514 PL 97 2471.

References


Table 3 Determination of sensitivity level of PCR for naturally occurring Cryptosporidium oocysts (A: sewage water, oocysts per 100 ml B: river water, oocysts per litre)

<table>
<thead>
<tr>
<th>No of oocysts by LSC*</th>
<th>150</th>
<th>121</th>
<th>120</th>
<th>105</th>
<th>100</th>
<th>96</th>
<th>85</th>
<th>78</th>
<th>66</th>
<th>36</th>
<th>28</th>
<th>26</th>
<th>22</th>
<th>12</th>
<th>8</th>
<th>6</th>
<th>4</th>
<th>3</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kind of sample</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>PCR</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Nested PCR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Nested PCR for C. parvum</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

* Laser Scanning Cytometry