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PCR and Real Time PCR for the Detection of *Cryptosporidium parvum* Oocyst DNA*

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Three DNA extraction kits were used, all without preliminary procedures, then DNA extraction was preceded with freeze/thaw cycles in three versions. A lack of desired effect resulted in the application of liquid nitrogen/water bath cycles before the use of the extractions in further experiments. The effectiveness of DNA extraction was measured by PCR signal and C_T values of real time PCR. A comparison of the efficiency of various *Cryptosporidium parvum* undiluted oocyst treatments prior to DNA extraction with the use of three kits has shown that the best results were obtained after extraction of DNA with the QIAamp DNA Tissue Mini Kit (T kit), preceded by triple liquid nitrogen/water bath in 100°C for 2 minutes and with overnight proteinase K digestion. After extraction with the T kit, the detection limit was 50 oocysts per 200 μl when effectiveness was evaluated with PCR and 10 oocysts in the case of real time PCR.

Key words: *Cryptosporidium parvum,* DNA extraction, PCR, real time PCR.

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Several species of *Cryptosporidium* are enteric protozoa that cause significant adverse health effects in humans and domestic mammals (CRAUN *et al*. 2005). These parasites are widely distributed throughout the world, as are their transmissive stages, i.e., oocysts. *Cryptosporidium* is recognized in the developed world as a significant waterborne pathogen (SUNDERLANDA *et al*. 2007). *Cryptosporidium* oocysts are prevalent in surface waters as a result of fecal contamination from wild animals, livestock and human waste. The enduring nature of oocysts, resistance to conventional chlorination disinfection techniques, low infectious dose, and modes of transmission all cause the persistence of these parasites in the environment for extended periods of time (CAREY *et al*. 2004; SUNNOTEL *et al*. 2006a).

In order to improve monitoring of *Cryptosporidium* oocysts in water, the United States Environmental Protection Agency (USEPA) introduced the 1622 Method and then the 1623 Method for concentration and detection of *Cryptosporidium* oocysts and *Giardia* cysts in water samples. Both methods are used for determining the presence and concentration of (oo)cysts in water, and they consist of: filtration, concentration, immunomagnetic separation (IMS), contrast staining and microscopic detection, as well as calculation of (oo)cyst numbers obtained by the methods. Currently, IMS and culture enrichment prior to DNA extraction of *Cryptosporidium* and *Giardia* are standard procedures in molecular methods (LOWERY *et al*. 2001; FONTAINE & GUILLOT 2003). However, the use of IMS is expensive, and it reduces the use of samples mostly to single organism detection (JIANG *et al*. 2005). The IMS is not only expensive; its performance is affected by the type of commercial kit, pH, and dissociation procedures (WARE *et al*. 2003). Thus, the development of methods for direct extraction of PCR quality DNA is very significant for the detection of pathogens in environmental samples.

The advent of molecular techniques, in particular those based on the *in vitro* amplification of nucleic acids, has improved our ability to detect

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infections caused by parasites. However, most diagnostic methods used in clinical practice have limited application with reference to the detection of protozoa in water samples. The most important restriction is a significantly lower concentration of cysts or oocysts in water in comparison with material taken from patients. For example, the levels of *Cryptosporidium* oocysts and *Giardia* cysts in surface water are very low, ranging from 0.5 to 1,500 organisms in 100 liters of water (PAYMENT *et al*. 2000). Based on a mathematical model on data from the Milwaukee outbreak, HAAS and ROSE (1994) suggested that some people may develop cryptosporidiosis after ingestion of only a single oocyst. The detection of such low numbers of organisms is difficult and requires very sensitive techniques.

Numerous direct DNA extraction methods have been tested in the preparation of DNA from *Cryptosporidium* spp. We have tried sonication, freezing in liquid nitrogen and boiling in a water bath. All of these procedures preceded the usage of three commercial kits for the IMS free DNA extraction of *C. parvum* oocysts. The effectiveness of DNA extraction was measured by PCR signal and a single – tube nested real time PCR targeting a specific region of rDNA of *C. parvum*.

Material and Methods

Purified bovine derived preparations of *Crytosporidium parvum* oocysts (Iowa strain) were obtained from Bulk Stock Live, BTF Biomérieux, Australia. Samples containing 5 x 10^4 oocysts of *C. parvum* with PBS in volume of 1 ml were used for genomic DNA extraction.

For choosing the best method of preisolation treatment, samples were processed as follows:

1. no treatment
2. boiling (100°C/5 min) and freezing (-70°C/5 min) repeated 5 times
3. boiling (100°C/2 min) and freezing in liquid nitrogen (2 min) repeated 3 times
4. sonication (2 min) repeated 3 times

In order to determine the detection limit, samples containing 5 x 10^4 oocysts of *C. parvum* were diluted in distilled water to 1 x 10^3, 5 x 10^2, 1 x 10^2, 5 x 10^1 and 1 x 10^1 oocysts per 200 μl. Samples were subjected to a preliminary treatment by boiling (100°C/2 min) twice and freezing in liquid nitrogen (2 min). After preisolation, DNA was isolated with three kits:

1. QIAamp DNA Stool Kit (QIAGEN) – St kit; incubation with proteinase 70°C/10 min, 56°C/overnight
2. QIAamp DNA Tissue Mini Kit (QIAGEN) – T kit; incubation with proteinase 56°C/h, 56°C/overnight
3. FastDNA SPIN Kit for Soil (MP Biomedicals) – So kit; modified protocol, extraction carried out without FastPrep Instrument, incubation with proteinase 56°C /overnight.

The effectiveness of DNA extraction was evaluated with PCR and TaqMan real time PCR.

Nested PCR amplification

A highly polymorphic region of the 18S rRNA gene was amplified by nested PCR. The method involves the amplification of an approximately 1,325 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, primers CX1F 5'-TTCTAGAGCTAATACATGCG-3' and CX1R2 5'-CCCTAATCCTTCGAAACAGGA-3' (XIAO *et al*. 1999) were used. Each PCR mixture (total volume 10 μl) contained 1 μl of Sigma (Germany) 10 × PCR buffer, 30 mM $MgCl_2$, 0.3 mM of each deoxynucleotide triphosphate, 5 pM of each primer, 5 U of Taq polymerase Sigma (Germany), and 1 μl of DNA template. For the second PCR step, we used 1 μl of the primary PCR product and primers CX2F 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and CX2R5' AAGGAGTAAGGAACAACCTCCA-3' (XIAO *et al*. 1999). 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*. (1999). Negative control reaction mixtures contained sterile distilled water in place of template DNA. PCR products were visualized by 1.5% agarose gel electrophoresis.

TaqMan nested real time PCR

A region of the small subunit rRNA gene of *C. parvum* was utilized as a target sequence for nested real time PCR. Primers CPrI and CPrII amplifying a 676 bp fragment (BIALEK *et al*. 2002) were used as outer primers. Inner primers, CPrF3 and CPrR3, amplifying 118 bp of the probe CPrP3-1, were designed by MINAROVICOVA *et al*. (2009). The PCR samples (total volume 10 μl) contained 800 nmol l^{-1} each of outer primers, inner primers and probe (all nucleotides were synthesized by Genomed, Poland), 375 μmol l^{-1} of each dNTP (Novazym, Poland), 2,5 mmol l^{-1} $MgCl_2$ and 1,5 U Taq DNA Polymerase (Sigma, USA). Reactions were performed in a Rotor Gene 6000 (Australia). Oligonucleotide sequences and the thermal profile were the same as described previously (MINAROVICOVA *et al*. 2009). A threshold cycle (CT) was calculated

for individual samples by the internal software of the cycler using the manual threshold setting at a fluorescence value of 10^{-2}. Appropriate negative controls were included in each PCR run. All analyses were carried out in four replicates.

Results

Three DNA extraction kits were used, all without preliminary procedures, then the DNA extraction was preceded with freeze/thaw cycles in three versions, but a lack of the desired effect resulted in the application of liquid nitrogen/water bath cycles before each extraction. A comparison of the efficiency of various *C. parvum* undiluted oocyst treatments prior to DNA extraction with the use of three kits, measured with nested PCR signal and C_T values of nested real time PCR, has shown that the best results were obtained after extraction of DNA with the T kit, preceded with triple liquid nitrogen/water bath in 100°C for 2 minutes and with overnight proteinase K digestion (Table 1).

The detection sensitivity was determined by analyzing two series of diluted suspensions of *C. parvum* oocysts in ten replicates. The detection limit, defined as the lowest number detected in 100% of cases, was 50 oocysts per 200 μl when effectiveness was evaluated with PCR and 10 oocysts with real time PCR after extraction with the T kit (Table 2, Figs 1, 2, 3). The detection limit after DNA extraction with two other kits was significantly higher (50 for St kit and 500 for So kit measured by real time PCR).

Table 1

Comparative efficiency of various *C. parvum* oocyst undiluted treatments prior to DNA extraction with the use of three kits, measured by PCR signal of an amplified 18S rRNA gene fragment and Threshold cycle (C_T) of real time PCR

Kit	Proteinase	Results of nested-PCR				Results of TaqMan real time PCR (threshold cycle – C_T)			
		No treatment	Boiling + freezing	Boiling + liquid nitrogen	Sonication	No treatment	Boiling + freezing	Boiling + liquid nitrogen	Sonication
St	70°C/10min	–	–	–	–	–	–	44.8 ± 0.35	–
	56°C/night	–	–	++++	+++	–	–	8.3 ± 0.82	19.8 ± 0.22
T	56°C/3 h	–	–	–	–	–	–	38.4 ± 0.17	46.8 ± 0.76
	56°C/night	–	–	++++	+++	–	–	4.1 ± 0.25	9.7 ± 0.86
So	56°C/night	–	–	–	–	–	–	22.7 ± 0.54	36.2 ± 0.11

++++ very strong intensity of the DNA band as determined by ethidium bromide stained-agarose gel electrophoresis
+++ strong intensity of the DNA band
– no DNA band visualized on ethidium bromide stained-agarose gel electrophoresis.

Table 2

The detection level of *Cryptosporidium parvum* oocysts in water suspensions defined by PCR signal and threshold cycle (C_T) of real time PCR with the use of three DNA extraction kits. Data represent mean +/– standard deviation for positive samples

Kit	Results of nested-PCR						Results of TaqMan real time PCR (threshold cycle - C_T)					
	Number of oocysts						Number of oocysts					
	$1x10^3$	$5x10^2$	$1x10^2$	$5x10^1$	$1x10^1$	Negative control	$1x10^3$	$5x10^2$	$1x10^2$	$5x10^1$	$1x10^1$	Negative control
St	++	–	–	–	–	–	28.5±0.12	34.4±0.45	39.1±0.35	43.9±0.27	–	–
T	+++	+++	++	+	–	–	11.5±0.93	26.4±0.54	35.3±0.23	39.8±0.87	46.6±0.63	–
So	–	–	–	–	–	–	36.4±0.17	42.3±0.28	–	–	–	–

+++ strong intensity of the DNA band
++ medium intensity of the DNA band
+ weak intensity of the DNA band
– no DNA band visualized on ethidium bromide stained-agarose gel electrophoresis.

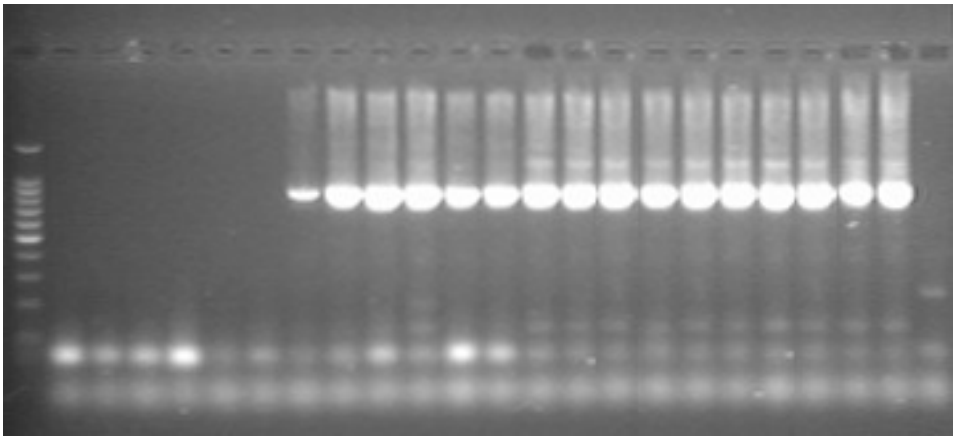

Fig. 1. Amplification products of a gene fragment encoding 18S SSU rRNA of *Cryptosporidium parvum* obtained from DNA isolated with the QIAamp DNA Tissue Mini Kit (Qiagen). M – molecular mass marker, paths 1-4 – amplicons obtained from a DNA isolate from 10 oocysts, paths 5-8 – amplicons obtained from a DNA isolate from 50 oocysts, paths 9-12 – amplicons obtained from a DNA isolate from 100 oocysts, paths 13-16 – amplicons obtained from a DNA isolate from 500 oocysts, paths 17-20 – amplicons obtained from a DNA isolate from 1000 oocysts, paths 21 and 22 – amplicons obtained from positive controls, path 23 – negative control.

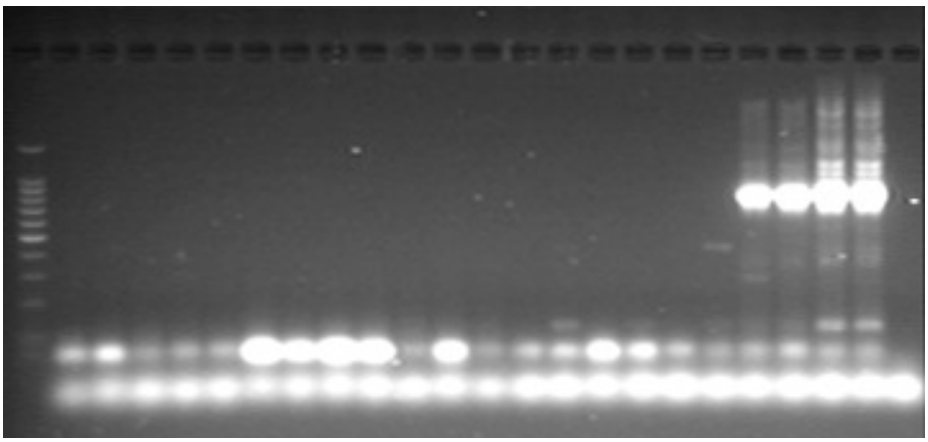

Fig. 2. Amplification products of a fragment of the gene encoding 18S SSU rRNA of *Cryptosporidium parvum* obtained from DNA isolated with QIAmp DNA Stool Kit (Qiagen). M – molecular mass marker, paths 1-4 – amplicons obtained from a DNA isolate from 10 oocysts, paths 5-8 – amplicons obtained from a DNA isolate from 50 oocysts, paths 9-12 – amplicons obtained from a DNA isolate from 100 oocysts, paths 13-16 – amplicons obtained from a DNA isolate from 500 oocysts, paths 17-20 – amplicons obtained from a DNA isolate from 1000 oocysts, paths 21 and 22 – amplicons obtained from positive controls, path 23 – negative control.

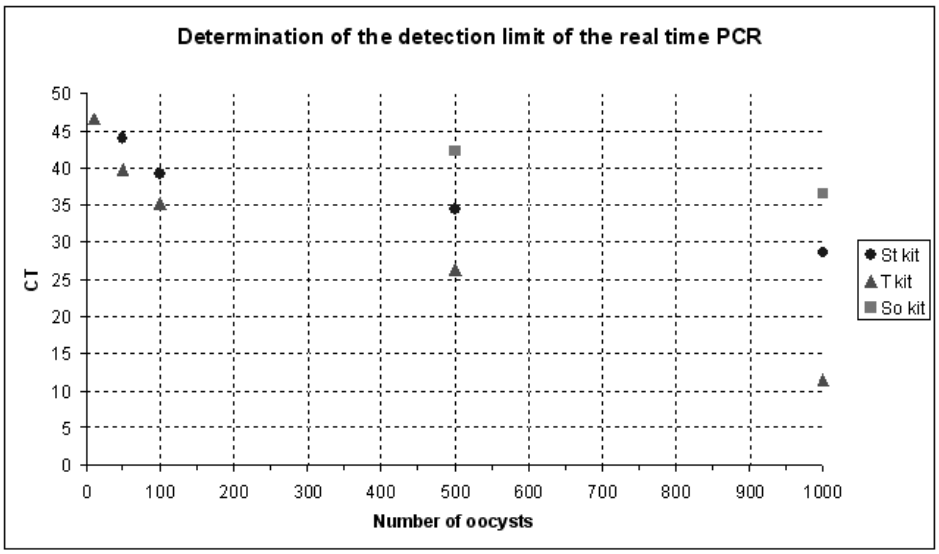

Fig. 3. Determination of the DNA-based detection limit of real time PCR after application of three DNA extraction kits. Average of triplicate threshold cycles (C_T) values +/– standard deviation are presented in most cases.

Discussion

Obtaining a high quality DNA extract is a basic step in PCR detection of pathogens in environmental samples, especially in the case of *Cryptosporidium*. Because *Cryptosporidium* oocysts occur in very low concentrations in water samples, the recovery of *Cryptosporidium* DNA during DNA extraction becomes important (JIANG *et al*. 2005). In addition, the exceptionally robust nature of the oocyst cell wall requires more stringent treatments for disruption. The disulfide bond – rich oocyst wall provides a protective barrier for infective sporozoites (MITSCHLER *et al*. 1994). The oocyst wall of *Cryptosporidium* spp., as usual for coccidians, is bilayered, consisting of a distinct outer and inner layer. This structure represents a unique feature of *C. parvum* and *C. hominis* oocysts. The outer layer of the oocyst wall is composed of acidic glycoproteins and is at least partially removed by treatment with sodium hypochlorite (REDUKER *et al.* 1985). It has been suggested that the elasticity and stability of the oocyst wall is a consequence of the central glycolipid/lipoprotein layer, and the large inner filamentous layer, which is composed of glycoproteins (BONNIN *et al*. 1991). HARRIS & PETRY (1999) examined the oocyst wall structure and its susceptibility to various treatments. A filamentous array on the inner surface was seen by transmission electron microscopy and this array can be depleted by digestion with proteinase K and trypsin, but pepsin was less successful. Ultrasonication of untreated oocyst walls produced almost no lysis. Treatment with proteinase K was the most effective in releasing the internal fibrillar layer from the oocyst wall, as compared to treatments with trypsin and pepsin. In these experiments chloroform treatment and phenol extraction did not disrupt the oocyst wall. A comparative study by SLUTER *et al*. (1997) of common DNA extraction techniques found that proteinase K treatment was no better than freezing in dry ice – ethanol and thawing in a 37°C water bath. Three cycles of freezing/thawing were retained as the optimal disruption method in these experiments. The only method yielding results comparable to freezing/thawing was sonication; freezing/thawing was selected over sonication for subsequent probes because it does not require access to specialized apparatus (SLUTER *et al*. 1997).

During the last few years, many studies describing the effectiveness of different treatments against the *Cryptosporidium* oocyst wall have appeared. However, the methodology has not been standardized yet. As a result, each laboratory undertaking this type of research has to carry out adaptation tests of techniques described in the literature in order to match them with their own conditions. Our first trials were carried out on clean oocysts of *C. parvum* in order to obtain a PCR signal. Three DNA extraction kits were used, all without preliminary procedures, then the DNA extraction was preceded with freeze/thaw cycles in three versions, but a lack of desired effect resulted in the application of liquid nitrogen/water bath cycles before the extractions were used in further experiments. A comparison of the efficiency of various *C. parvum* undiluted oocyst treatments prior to DNA extraction with the use of three kits, measured with nested PCR signal and C_T values of nested real time PCR has shown that the best results were obtained after extraction of DNA with the T kit, preceded with triple liquid nitrogen/water bath in 100°C for 2 minutes and with proteinase K digestion overnight (Table 1). Satisfying results have also been also obtained after extraction with the St kit preceded by a triple liquid nitrogen/water bath for 2 minutes in 100°C and with proteinase K digestion overnight. However, a comparison of C_T values of real time PCR after extraction with the T kit and St kit, with the assumption that the higher initial number of DNA copies, the smaller C_T values, has shown that the effectiveness of the first kit was higher (Table 1). Satisfying results have been also obtained when the extraction of DNA was preceded by sonication, but the PCR signal in that case was weaker and C_T values of real time PCR were higher than those obtained after DNA extraction preceded with liquid nitrogen/water bath (Table 1). DNA extraction with the So kit did not result in the expected amplification products, but only in the real time PCR, which was probably caused by introduced modification (Table 1). The producer recommends a very expensive FastPrep Instrument for homogenization of samples, but this was not used in the presented study, instead overnight digestion with proteinase K was applied.

The effectiveness of DNA extraction with the use of each Mini Kit after extraction with each treatment was measured by nested PCR and C_T values of nested real time PCR of the SSU rRNA gene of *C. parvum.* The detection limit, defined as the lowest number detected in 100% cases, was 50 oocysts per 200 μl when effectiveness was evaluated with nested PCR and 10 oocysts with nested real time PCR after extraction of DNA with T kit (Table 2). Using 18S rRNA as a target, the detection sensitivities of real time PCR and nested PCR systems were compared by SUNNOTEL *et al*. (2006b). The sensitivity analysis of this assay determined that it was routinely capable of detecting three oocysts.

When the highest sensitivity of PCR is required, nested PCR, involving two reactions, is often used. The product of the first reaction is used as a template for the second reaction. In this way, all irregularities should take place in the first reaction. The second one, starting amplification from a rea-

sonably high amount of the template, should be already fully effective. Sensitivity of detection of *C. parvum* has been further improved by using nested PCR (MONIS & SAINT 2001; ESSID *et al*. 2008). Unfortunately, conventional nested PCR is not very suitable for routine diagnostics usage because of the risk of contamination by short amplified DNA fragments (ABRAVAYA *et al*. 1997). This risk can be eliminated by applying single-tube nested PCR, where two pairs of primers are applied with different melting points and annealing temperatures in a first and second PCR reaction. In the presented work, we have applied a single-tube nested real time PCR protocol for the detection of *C. parvum*, the development of which was carried out by MINAROVICOVA *et al*. (2009). It is a version of the single-tube nested PCR, where the second PCR round employs a 5'-nuclease TaqMan probe. Thus, the second – round PCR can be monitored continuously, in closed tubes, without the need of any subsequent, discontinuous analysis. This version of the method seems to be very useful for the sensitive detection of DNA of *C. parvum*, whereas results of our comparative studies have shown that all stages preceding the molecular detection of *C. parvum* are equally important and influence the final effect.

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