IDENTIFICATION OF *CRYPTOSPORIDIUM SP. OOCYSTS* IN WATER BY MODIFIED IMMUNOMAGNETIC SEPARATION AND RFLP-PCR

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ABSTRACT
Current methodologies for detection of *Cryptosporidium sp*. in water, despite some efforts for standardization (e.g. EPA Methods 1622/1623), has several drawback that limit the applicability or interpretation of the results of *Cryptosporidium* monitoring. Presence of some chemical dissolved in water, ageing of oocysts, reduced affinity of antibodies used in immunomagnetic separation (IMS) and immunofluorescent assay (IFA) results in reduced sensitivity and specificity. Against this background of molecular biology techniques used for detection of *Cryptosporidium*, are characterized by good sensitivity, reproducibility and acceptable cost analysis.

In this paper, the authors showed the possibility of modifying the protocol of immunomagnetic isolation of oocysts and adjust its scale to the needs of a PCR reaction combined with an analysis of restriction fragments length polymorphism (RFLP-PCR). The method allows relatively rapid implementation of qualitative analysis in the direction of *Cryptosporidium*, combined with the identification of species. It is also connected to the determination of several pathogens (including *Giardia* and *Toxoplasma*) through a combination of antibodies used in the IMS and multiplex-PCR.

INTRODUCTION

Cryptosporidium is a small, obligate intracellular protozoan parasite that infects epithelial cells in the digestive tract of numerous vertebrates including human and other mammals, birds, reptiles and fish. The parasite form thin-walled or thick-wall oocysts that are relatively small (4-6µm), nearly spherical and have few or no diagnostic features to identification [Marshal et al, 1997]. Thin-wall oocysts may excyst within the same host, resulting in severe diarrhoea while thick-wall oocysts are shed by infected hosts into the environment where are able to survive under adverse conditions for a long time until ingested by a new host [Fayer et al, 2000]. Original description of *Cryptosporidium* was given in the beginning of twentieth century [Tyzzer, 1907], but first infection with *Cryptosporidium* was reported only just in 1976 and in following 30 years there were approximately 150 described cases of cryptosporidiosis from over 90 countries [Smith & Rose, 1998; MacKenzie et al, 1994].

The need for water analysis for the presence of dangerous to public health, intestinal parasitic protozoa is becoming stronger, as the number of waterborne worldwide outbreaks of cryptosporidiosis is still growing.In response to the identified risk to public health, the U.S. Environmental Protection Agency (EPA) in the period 1996 1999 has introducedstandard protocols for detection of *Cryptosporidium parvum*oocysts in water (EPA, 2001[a]; EPA 2001[b]). These protocols (method 1622 and 1623) are based on a scheme involving isolation of oocysts from the water, their marking and counting using fluorescence microscopy.The main aim of these protocols

is to determine the number of viable, capable of infection, *Cryptosporidium*oocysts. Technically, EPA protocols are different from the classical methods of quantitative determination of indicator bacteria in water, and involve four stages: collecting samples of water and its concentration, immunomagneticseparation of oocysts from other components of the suspension (IMS), labeling of oocysts monoclonal antibodies labeled with a fluorescent marker (IFA) and the counting of oocysts in a DIC/fluorescent microscope [DiGiovani et al, 1999; Jenkins et al, 1997; Robertson et al, 1998].The protocols primary developed by EPA have been the subject of numerous modifications, however, despite the numerous effortscurrently available procedures remain still uncertain - the recovery of oocysts on the stage of isolation is low and variable, there is no possibility of an assessment of viability of oocysts or the ability to identify species/genotype, and thus assess whether the found oocysts are infectious to humans. Total recovery of oocystson the stage of filtration/IMS depends on so many different factors that it is virtually impossible to compare the results obtained in different laboratories, despite efforts to standardize [Clancy, 2001].

RFLP-PCR combines the analysis of DNA restriction fragment length polymorphism in relation to the PCR product and is used, with some success in experimental works, to examine the species of the genus *Cryptosporidium*. Successful choosing the pair of PCR primers that yielded a fragment of gene duplication within the observed length polymorphism and sequence polymorphism allows to achieve the sensitivity characteristic of the PCR with the ability to identify certain species or even genotypes [Coupe et al 2005, Nichols et al 2003, Lenget al 1996, Azamiet al 2007, Yang et al 2008]. All stages of analysis (DNA extraction and purification, PCR, digestion with restriction enzymes and electrophoresis) are relatively simple and allow to detect as little as 10oocysts within few hours [Xiao et al., 2001; Polus&Kocwa-Haluch, 2009].

Experimental studies have shown successful use of restriction polymorphism within the gene for 18S rRNA for detection and differentiation of *C. parvum*, *C. hominis*, *C. muris*, *C. andersoni*, *C. felis*, *C. baileyi*, *C. meleagridis*, *C. serpentis* and *C. wrairi*[Fayer et al, 2000; Xiao &Fayer, 2008].Successive digestion of PCR products with appropriately selected restriction enzymes can generate restriction fragments of different lengths and differentiate all these species of Cryptosporidium [Johnson et al, 1995, Nichols et al, 2003, Xiao et al, 1999].At the moment there is no standard locus for the study of RFLP, but with all the known loci, nevertheless polymorphism within the small 18S subunit of rRNA gene carries information to distinguish the largest number of *Cryptosporidium* species.Certainly it can be concluded that although PCR-RFLP does not identify all the species or genotypes of *Cryptosporidium*, it can differentiate all the species and genotypes important for public health.

The purpose of this study to develop a possible simple and rapid method of DNA isolation and purification from oocysts of *Cryptosporidium sp*. and apply RFLP-PCR for both, sensitive detection of *Cryptosporidium*, and identification of given specie. Since differences in the length of RFLP fragments are slight but still perceptible in agarose gel electrophoresis, it was necessary to very precisely determine the test conditions to eventually detect the presence of the pathogen in water.

METHODS

Sample preparation
Water samples (5-50 liters) were subjected to membrane filtration on cellulose esters filters (Millipore RAWP/SSWP, diameter 90mm, pore size 1,2-3,0 µm). An important factor limiting the filtration was amount of suspension present in the water. Very turbid water samples were subjected to preliminary pre-filtration through glass fiber filters (Millipore, APFD). In this case, a decrease in recovery of oocysts was offset by a much larger volume of sample that could be analyzed. The

material gathered on filter was gently scraped and suspended in cold 0,01% Tween-20 in PBS to final volume of 20ml. The suspension was layered on the aqueous solution of sucrose (d=1,17 g/ml) and centrifuged (1250xg, 15 minutes, 4°C). The viscid interphase was collected and washed twice in cold PBS. Concentrated material was used for immunomagnetic separation of *Cryptosporidium*oocysts.

IMS was adopted from the manufacturer protocol (Invitrogen, Dynabeads® Anti Cryptosporidium Kit),however the scale of the original procedure was too large for PCR needs (moreover there is no need to remove paramagnetic beads before isolation of DNA), therefore some modification was applied. The most important is the abandonment of a standard MPC magnet (Dynal) in favor of smaller magnets (Applied Biosystems 6 Tubes Magnetic Stand), adapted to the standard 1,5 ml test tubes to simplify the further steps of separation. In brief, pellets gathered in earlier steps were suspended in 1ml of 1x SLA buffer (100µl of 10x SLA and 100µl of SLB in 1ml of water) subsequently, addition of 10µl of anti-Cryptosporidium Dynabeads® was made. Then the tubes were placed horizontally on a shaker plate and subjected to stirring for 3 hours. After the shaking test tubes were placed in a magnetic rack, slide the magnet and performed gentle swinging motion for 2 minutes, until the suspension of magnet beads assembly to the wall of the tube. Buffer was discarded and the remaining beads were rinsed twice in 1ml of 1x SLA buffer and subsequently in cold PBS.

As a control procedure for isolation of material from the water samples, particularly to evaluation of the IMS procedure recovery, the control seeds of *Cryptosporidium parvum*oocysts (P102C, Waterborne Inc.) were made.The water samples negative for Cryptosporidium (total volume: 5-10 liters) were inoculated 10 to 10,000 *C. parvum*oocysts and filtration/IMS was performed as described above. The collected material was used for DNA isolation and PCR.

DNA extraction

The pellets collected in preceding step were suspended in 900µl of lysis solution (10mM Tris-HCl [pH 8,0], 1mM EDTA, 0,5% sodium dodecyl sulphate) and subjected to 10 freeze-thaw cycles (1min in liquid nitrogen and 2min in 60°C per cycle) [Nichols et al, 2003]. Samples were then incubated for 12 hours (overnight) with proteinase K (200µg/ml, 55°C) while being vigorous shaking in thermomixer. After this time tubes were incubated for 20min in 90°C to denaturate proteinase K and triple extraction with same volume of phenol:chloroform mixture (1:1 v/v, pH 8,0) [Sambrook&Russel, 2001]. The latter extraction was subsequently followed by precipitation with 1,5 volume of ice-chilled isopropanol. The samples were overnight stored in freezer (-20°C) and then centrifuged (30min, 15000xg, 4°C). Pellets were once washed with 70% ethanol, air-dried and suspended in 200µl of molecular biology grade water.

Alternatively DNA was extracted by QIAamp DNA Stool Kit (Qiagen), however the original protocol was subject of few modifications. The pellets collected in preceding step were resuspended in 1400µl of bufer ASL. The suspension was heated to 95°C for 10min, vortexed and centrifuged (1min, 15000xg). Supernatant was treated with InhibitEX according to manufacturer's recommendations. Digestion with proteinase K was elongated to 30min at 70°C. All subsequent steps were carried out in accordance with original protocol.

Purification of obtained DNA samples were completed using High Pure PCR Template Preparation Kit (Roche) according to manufacturer's protocol.

RFLP-PCR

All PCR amplification were performed in a Eppendorf Personal MasterCycler in 0,2ml thin-wall tubes. Reaction volumes of 25µl consisted premixed reagents containing a 250µM of each of the dNTPs (Fermentas), 0,5µM of each of primers (forward: aagctcgtagttggatttctgreverse: taaggtgctgaaggagtaagg), 0,75U of Taq DNA polymerase (Biotools) and 1µl of sample.

Concentration of MgCl$_2$ in working solution was set to 1,5mM according to manufacturer's recommendations. Positive and negative controls for each reaction included 1µl template consisting of water with and without 100pg of *C. parvum* genomic DNA (PRA-67D, ATCC). Samples were incubated in thermocycler with the following programmed profile: initial denaturation for 3min at 95°C and 40 cycles of amplification (denaturation for 45s at 94°C, primers annealing for 45s at 54°C and elongation for 75s at 72°C) [Rochelle et al, 1997; Johnson et al, 1995]. The final extension was set to 10min at 72°C, and then samples were cooled to 4°C.

In the present study FastDigest® (Fermentas) restriction enzymes were used (*DdeI*, *DraI*, *VspI*).Use of such enzymes can reduce the digestion of the typical several-dozen hours to just 15 minutes. Reaction profile included the digestion phase (37°C, 15 minutes), phase of thermal inactivation of the enzyme (65°C, 5 min) and final cooling (4°C hold). Restriction enzyme reaction mixture was prepared immediately after the PCR reaction, using the fourth volume of the PCR product (10-12µl) to cut.After heat inactivation of FastDigest® enzymes electrophoresis of digestion products was completed in 3% gel (2% standard agarose and 1% agarose LMP, in TAE) stained with 1x SYBR Safe DNA Gel Stain.Since the enzymes used to generate restriction fragments of different length, so the resulting image of the restriction fragments, allow to identify the most species of the genus *Cryptosporidium*(Table 1).

RESULTS AND DISCUSSION

The presented results show that it is possible to use molecular biology methods for detection of *Cryptosporidium sp.* oocysts in environmental samples such as surface water. PCR itself has a very good sensitivity of 0,1pg of genomic DNA, which permit seamless detection of 10 copies of genomic DNA [Abrahamsen et al, 2004] means, in practice the ability to detect 2-3 *Cryptosporidium sp.* oocysts, assuming no losses to isolate DNA (Fig. 1). This sensitivity means that the additional treatments in the form of an internal PCR, or detection of products by dot blot hybridization, are not required to obtain satisfactory results.In other words, this technique allows to capture the presence of *Cryptosporidium sp.* oocysts in water already at a concentration of about 0,5-2 per liter.

Protocol presented in this paper seems to be greatly simplified. Further oversimplification, including the elimination of time-consuming steps such as freezing in liquid nitrogen (Fig. 2), or elimination of IMS phase (Fig. 3), lead to significant declines sensitivity. It seems that the elimination of the IMS leads to a particularly uncertain results, in spite of so considerable cost of this stage, it must be maintained.

If the test material (i.g surface water or other environmental samples) contain *Cryptosporidium sp.* oocyst and DNA extraction and purification were done correctly, the PCR products are formed with a length characteristic of the given *Cryptosporidium* species. Differences in the length of the individual products are not large enough to be able to make on their basis identification of species. Only RFLP generates fragments of visible different length so that the agarose electrophoresis is sufficient to identify them. On the following figures (Fig. 4-6) are shown the results of RFLP performed on material isolated from samples containing successively *C. parvum*oocysts (Fig. 4), *C. muris*oocysts (Fig. 5) and *C. meleagridis*oocysts (Fig. 6). As is shown, demonstration of the presence of two commonly occurring *Cryptosporidium*species, and their distinction is not problematic.

The simultaneous presence of oocysts of several *Cryptosporidium* species in water, could makesome difficulty, arising from the very nature of PCR. Although as is shown, it is possible to simultaneously detect and identify two species, if RFLP restriction fragments give significantly different in length, and when the concentration of oocysts comparable (Fig. 7).However, you can expect that when these conditions are not met only one species present in the waterwill be

detected.So it is possible to mask the presence of such species infective to humans by the presence of species which did not create any hazard to humans.

In summary we can say that the PCR-RFLP is a promising technique capable for detection and identification of *Cryptosporidium sp.* oocysts in variety of environmental water samples. In comparison to the classic 1622/1623 EPA protocols, it brings certain advantages (speed, sensitivity, species specificity, relatively low price), but not without shortcomings, eg it not possible to assess oocyst viability.

Images

Table 1. Lengths of PCR products and restriction fragment length for selected species of *Cryptosporidium* genus.

|		PCR	restriction fragment length [bp]		
		[bp]	*DdeI*	*DraI*	*VspI*
1	*C. parvum*	435	201, 166, 68	---	219, 112, 104
2	*C. hominis*	438	204, 166, 68	---	222, 112, 104
3	*C. muris*	432	224, 166, 42	---	320, 112
4	*C. andersoni*	431	265, 166	---	319, 112
5	*C. felis*	455	221, 166, 68	50, 405	239, 112, 104
6	*C. baileyi*	428	262, 166	84, 344	212, 112, 104
7	*C. meleagridis*	434	200, 166, 68	---	171, 112, 104, 47
8	*C. serpetis*	430	264, 166	---	318, 112
9	*C. wrairi*	435	201, 166, 68	---	219, 112, 104
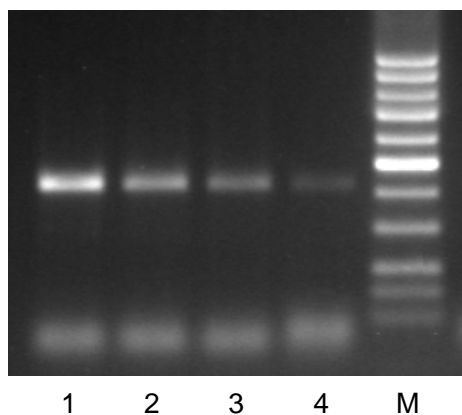
Figure 1. Sensitivity of PCR reaction expressed as pg/fg of *C. parvum*DNA capable of detecting. 1 - 100pg, 2 - 10pg, 3 - 1pg, 4 - 100fg, M - DNA ladder 100bp.
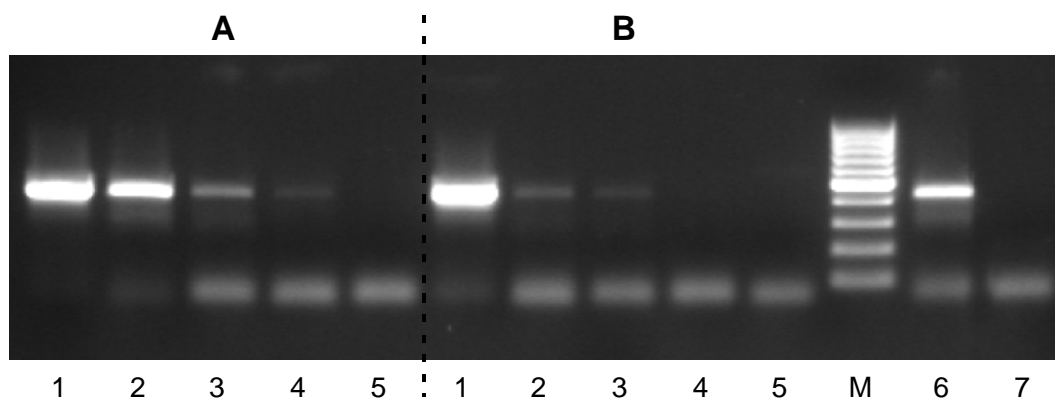
Figure 2. Comparison of DNA extraction performed using freezing in liquid nitrogen (A) or without freezing (B) of the control seeds of *C. parvum*oocysts in water. The number of oocysts used: 1 - 10000 oocysts, 2 - 1000 oocysts, 3 - 100 oocysts, 4 - 10 oocysts, 5 - 1 oocyst, 6 - positive control, 7 - negative control, M - DNA ladder 100bp.
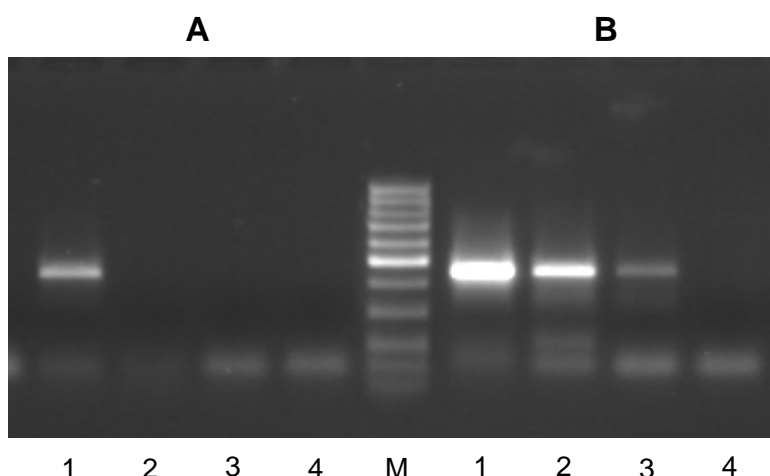

Figure 3. Comparison of the impact of IMS for extraction of DNA from *C. parvum*oocysts. Extraction of DNA was done by SDS lysis, freezing in liquid nitrogen and digestion with proteinase K. A - DNA extraction without IMS, B - DNA extraction accomplished after IMS. The number of oocysts used: 1 - 5000 oocysts, 2 - 500 oocysts, 3 - 50 oocysts, 4 - 5 oocysts, M - DNA ladder 100bp.
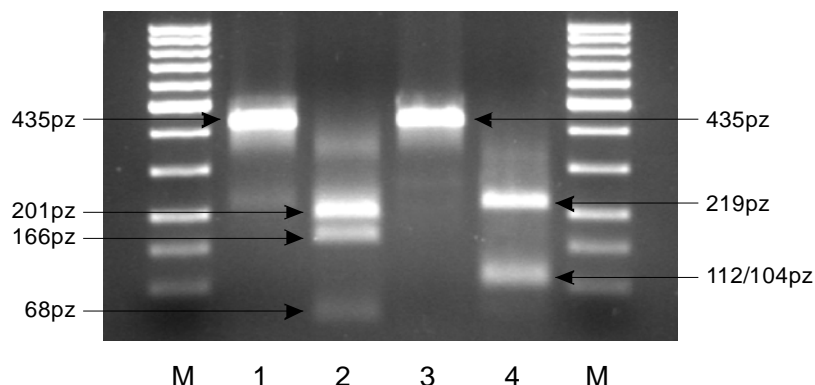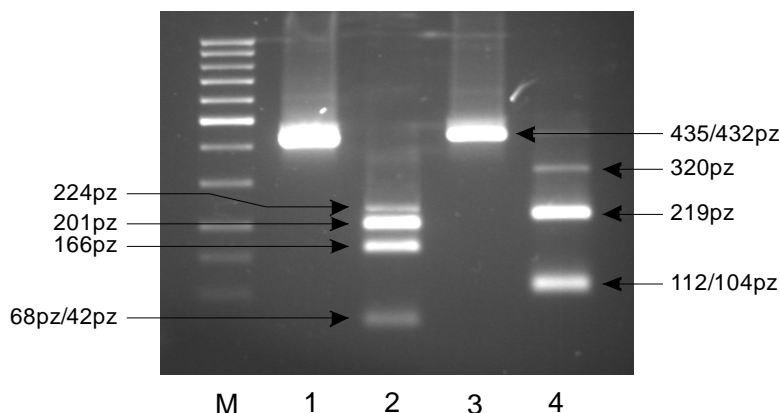
Figure 4. Electrophoretic image obtained after digestion with restriction enzyme amplification product of DNA isolated from *C. parvum*oocysts. 1 - undigested PCR product, 2 - *DdeI* enzyme digestion, 3 - *DraI* enzyme digestion, 4 - *VspI* enzyme digestion , M - DNA ladder 100bp.
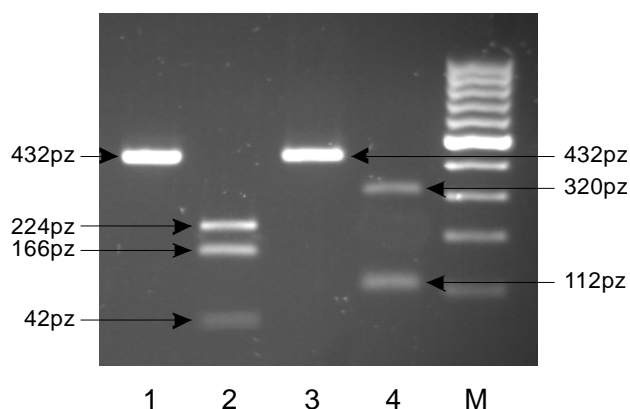
Figure 5. Electrophoretic image obtained after digestion with restriction enzyme amplification product of DNA isolated from *C. muris*oocysts. 1 - undigested PCR product, 2 - *DdeI* enzyme digestion, 3 - *DraI* enzyme digestion, 4 - *VspI* enzyme digestion , M - DNA ladder 100bp.
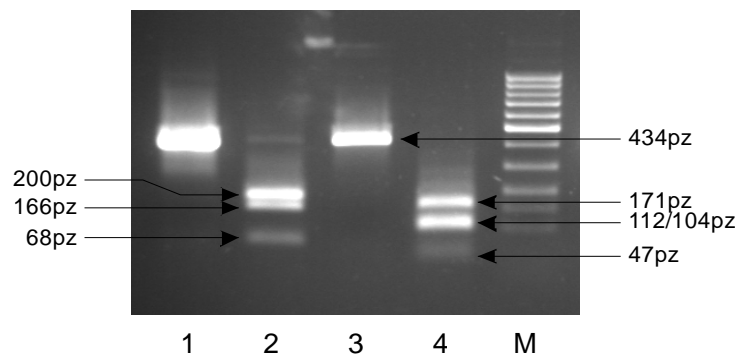
Figure 6. Electrophoretic image obtained after digestion with restriction enzyme amplification product of DNA isolated from *C. meleagridis*oocysts. 1 - undigested PCR product, 2 - *DdeI* enzyme digestion, 3 - *DraI* enzyme digestion, 4 - *VspI* enzyme digestion , M - DNA ladder 100bp.

Figure 7. Electrophoretic image obtained after digestion with restriction enzyme amplification product of DNA isolated from material containing *C. parvum* and *C. muris*oocysts. Shown bands have a characteristic length for both species. 1 - undigested PCR product, 2 - *DdeI* enzyme digestion, 3 - *DraI* enzyme digestion, 4 - *VspI* enzyme digestion , M - DNA ladder 100bp.

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