A sensitive, semi-quantitative direct PCR-RFLP assay for simultaneous detection of five *Cryptosporidium* species in treated drinking waters and mineral waters

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Abstract We describe a semi-quantitative PCR-RFLP method for detecting low densities of *Cryptosporidium* spp. oocysts present in final drinking water samples and natural mineral waters. UK Standard Operating Protocols were used to concentrate oocysts from drinking water samples. Oocysts were concentrated from mineral waters by membrane filtration. *Cryptosporidium* oocysts identified by epifluorescence microscopy on slides or filters were subjected to DNA extraction and PCR-RFLP analysis. Oocysts were disrupted by freeze-thawing in lysis buffer. Amplicons were readily detected from 2 to 5 intact oocysts on ethidium bromide stained gels following 1 round of PCR. DNA extracted from *C. parvum*, *C. muris*, *C. baileyi*, human-derived *C. meleagridis*, and *C. felis* were used to confirm species identity by PCR-RFLP following simultaneous digestion with *DraI* and *VspI*.

Keywords *Cryptosporidium* spp. oocysts; drinking water; mineral water; semi-quantitative direct PCR-RFLP; simultaneous detection

Introduction

The coccidian parasite, *Cryptosporidium parvum*, infects the enterocytes of numerous mammalian hosts including man, causing self-limiting diarrhoea in immunocompetent hosts and prolonged intractable diarrhoea in immunocompromised hosts. Human infection has been documented in both developed and developing countries (Fayer and Ungar, 1986) and economic conditions which result in poor sanitation and ineffective water treatment contribute significantly to the high incidence of infection and stunted growth in young children (Checkley *et al*., 1997; Molbak *et al*., 1997).

Cryptosporidium species, other then *C. parvum*, can also infect immunocompromised (Pieniazek *et al*., 1999; Morgan *et al*., 2000b) and immunocompetent individuals (Pedraza-Diaz *et al*., 2001; Xiao *et al*., 2001). Molecular analysis of oocyst DNA from these patients, by PCR-RFLP and by sequence of the PCR product obtained from multiple polymorphic genetic loci, revealed identity with *C. meleagridis* (15), *C. felis* (14), and *C. parvum* dog genotype (4). In immunocompetent individuals, Pedraza-Diaz *et al*. (1999), reported 6 of 1735 UK patients with cryptosporidiosis were infected with *C. meleagridis*. From 1090 *Cryptosporidium* positive faecal samples that were genotyped at the Scottish Parasite Diagnostic Laboratory (SPDL) only one individual harboured infection with *C. meleagridis* (unpublished data). Analysis of faeces from 80 immunocompetent Peruvian children (132 stool specimens, 29% with diarrhoea) revealed the presence of 5 *Cryptosporidium* genotypes (*C. parvum* genotype 1 (67), *C. parvum* genotype 2 (8), *C. parvum* dog genotype (2), *C. meleagridis* (7) and *C. felis* (1) (Xiao *et al*., 2001). *C. meleagridis* has also been found in an Indian ring-neck parrot (Morgan *et al*., 2000a) and *C. felis* in a cow (Bornay-Llinares *et al*., 1999).

Currently, molecular techniques are applied with greater frequency to detect oocysts in environmental water samples and food (Johnson *et al*., 1995; Kostrzynska *et al*., 1999;

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Lowery *et al*., 2000; Deng and Cliver, 2000; Xiao *et al*., 1998, 1999). IMS-PCR has been used to separate oocysts from inhibitory substances present in the samples, with reported detection sensitivity after DNA amplification of 10–100 oocysts in environmental water samples, apple juice or milk. Some of these assays are specific for *C. parvum* (Kostrzynska *et al*., 1999; Deng *et al*., 2000) while others are designed to speciate by IMS-PCR-RFLP (Xiao *et al*., 1999; Lowery *et al*., 2000).

The extent of the occurrence of species other than *C. parvum* in the environment is only now beginning to be addressed. Xiao *et al*. (2000) reported that of 29 storm water samples in the USA, *Cryptosporidium* spp. were present in 27 of them, mainly wildlife *Cryptosporidium* genotypes. The most common genotypes found in surface waters were *C. parvum* genotypes 1 and 2 and *C. andersoni*, with *C. andersoni* reported to be most commonly found in wastewater (8 samples). However, RFLP patterns indicated mixed populations and sequence analysis showed that only 4 genotypes had 100% homology with previously sequenced species.

Both sanitation and agricultural practices contribute to the contamination of the aquatic environment (Smith *et al*., 1995). Water is an important transmission route, and the environmental robustness of oocysts permits prolonged persistence in the aquatic environment (Smith and Rose, 1990; Tamburrini and Pozio, 1999). *Cryptosporidium* oocysts have been implicated in over 55 waterborne and foodborne outbreaks of cryptosporidiosis (Girdwood and Smith, 1999; Fayer *et al*., 2000; Slifco *et al*., 2000). Oocyst contamination of groundwater has also been reported (Anon, 1992; Hancock *et al*., 1998), and an outbreak of waterborne cryptosporidiosis, associated with a chalk borehole, with 345 confirmed cases of illness occurred in the UK (Willocks *et al*., 1998).

Natural mineral water sources are usually situated in protected sites and their exploitation is strictly regulated. This is a rapidly expanding industry. The consumption of bottled waters has increased in the UK from <5 million litres in 1975 (Stickler, 1989) to 1,380 million litres in 2000 (www.globaldrinks.com), comprising 67% natural mineral waters, 28% spring waters and 5% other waters) with the exploitation of numerous new sites. Worldwide, the consumption of mineral waters is high: 91 litres per annum per capita in Western Europe (www.zenithinternational.com). UK guidelines governing the microbiological quality of natural mineral waters (Anon., 1989) state that eukaryotic parasites should be absent, prior and during the water exploitation. However, a standard method for the detection of parasites in natural mineral waters is not available at present.

Here, we describe a semi-quantitative method for the detection and simultaneous species identification of small numbers of *Cryptosporidium* oocysts in drinking water concentrates and still, natural mineral waters by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP).

Materials and methods

Cryptosporidium oocyst sources

Human isolate oocysts, purified according to Bukhari and Smith (1995) were obtained from the SPDL oocyst isolate bank. Purified *C. parvum* oocysts, Moredun isolate, (MD, Edinburgh, UK) and the Iowa isolate (Pleasant Hill Farm (PHF), USA) were purchased. Both *C. muris* (RN 66) and *C. baileyi* (Belgium strain, LB 19) isolates were donated by Dr. K. Webster (VLA, Weybridge, UK). Purified *C. meleagridis* oocysts were obtained from the *Cryptosporidium* Reference Unit, Swansea PHL, UK and from the SPDL *Cryptosporidium* oocyst isolate bank. DNA extracted from oocysts isolated from a cat, presumptive *C. felis*, was donated by Dr. G. Lindegard, Cornell University, USA).

Oocyst recovery from final water samples

Final waters were sampled according to the Standard Operating Protocol identified in Part 2 of the Protocol for monitoring *Cryptosporidium* oocysts in water supplies (UK Drinking Water Inspectorate Information letter 10/99) generated to implement The Water Supply (Water Quality) (Amendment) Regulations 1999**,** SI No. 1524. A total of 9 *Cryptosporidium* oocyst positive samples were tested.

Mineral waters

Four commercially available, still natural mineral waters containing a range of total dissolved salts (TDS) (A = 91 mg l^{-1}, B = 136 mg l^{-1}, C = 280 mg l^{-1}, and D = 430 mg l^{-1}) were selected. Reverse osmosis water (RO) was the reference control. Mineral water samples and RO water were seeded with small numbers of oocysts (50 oocysts l^{-1}), filtered through flat bed membranes (Figure 1). Mock recoveries were performed with mineral water D and concentrates were seeded with small numbers of oocysts. DNA was extracted and diluted to determine PCR sensitivity.

DNA extraction

A freeze-thaw protocol for maximising DNA extraction from oocysts was followed (Nichols and Smith, submitted). DNA from oocyst isolates was extracted in either PCR or lysis buffer (LB) (Figure 2) by freezing and thawing. Oocysts disrupted in LB were digested in proteinase K. Following extraction in either PCR or LB, lysates were incubated (90°C, 20 min) to denature proteins associated with the nucleic acids or proteinase K, cooled on ice (1 min) and centrifuged (16,000 g, 5 min). Supernatants were either used immediately for PCR amplification or kept at –20°C until used.

PCR protocol

The method of Johnson *et al*. (1995) using the primers CPB-DIAGF and CPB-DIAGR were used. To detect oocysts seeded into mineral water concentrates, 50 µl of extracted DNA in PCR buffer was used in a total PCR reaction volume of 100 µL. To determine the limit of detection, volumes of extracted DNA ranging from 0.5–20 µl were used. To detect oocysts trapped on membrane filters, 5–10 µl of DNA extracted in LB was used per reaction.

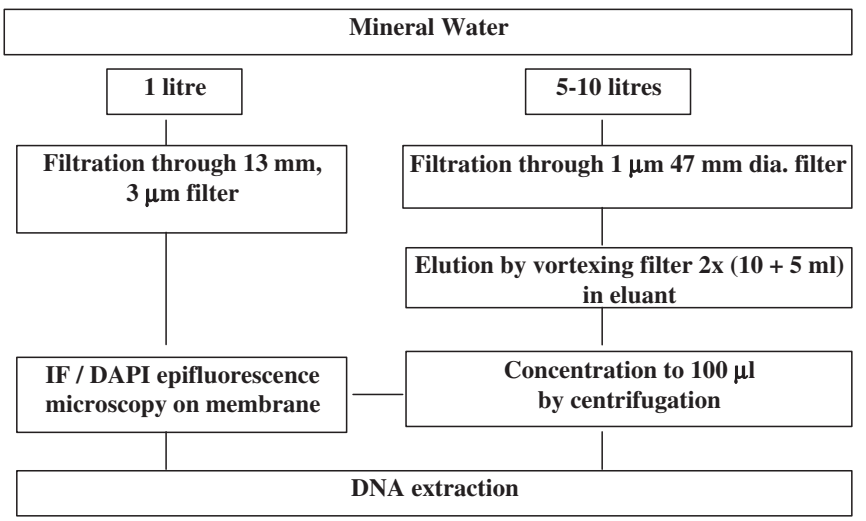

Figure 1 Schematic representation of filtration methods for concentrating and enumerating *Cryptosporidium* oocysts seeded in mineral waters, prior to nucleic acid extraction

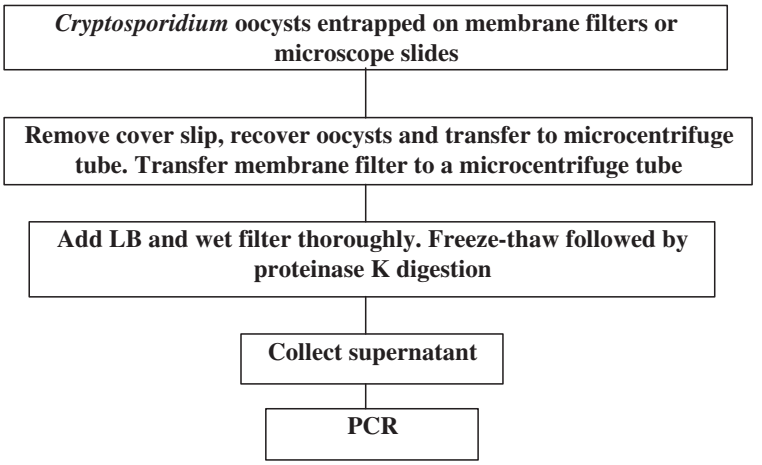

Figure 2 Schematic representation of DNA extraction steps applied to *Cryptosporidium* oocysts following IF/DAPI microscopy

Construction of competitor molecule for co-amplification with CPB-DIAGR/F primers

The internal control was constructed according to Ross *et al*. (1995). The strategy has the following requirements: 1) the control should be amplified with the same set of primers to minimise differences in amplification conditions; 2) the control and the target DNA amplified fragments will differ by less than 20% in product length to keep the same amplification efficiency; 3) the control DNA will have minimum identity with the target DNA to avoid hybrid formation, which happens especially after many rounds of amplification. For amplifying the *Cryptosporidium* 18S rRNA gene, the control internal sequence should not contain *DraI* or *VspI* restriction endonuclease sites in its sequence since we use these enzymes for species identification.

Species identification by PCR-RFLP analysis

Purified oocysts of *C. parvum* (human isolate), *C. muris*, *C. baileyi*, *C. meleagridis* and *C. felis* were used to confirm species identification by PCR-RFLP. Approximately 10^3 oocysts of each species, suspended in 100 µl of PCR buffer, were freeze-thawed and 20 µl of supernatant was used for PCR amplification. Ten µl of PCR product was digested (2 h, 37°C) with 10U each of *DraI* and *VspI* in 20 µl of React 1 buffer. Undigested controls, incubated without enzymes, were run alongside the digested fragments in a 1.4% agarose gel electrophoresis (100 V, 2 h) and stained with ethidium bromide (0.5 µg ml^{-1}).

Results and discussion

Cryptosporidium oocyst detection in final water samples

Oocysts (range 2–36) were detected in 8 of 9 water samples analysed by IF/DAPI microscopy. Seven samples were positive by PCR with the 18S rRNA gene primers and all 6 samples tested were positive with the single tube nested COWP assay (Homan *et al*., 1999). RFLP analysis of the PCR product indicated that *C. parvum* genotype 1 was the only *Cryptosporidium* species detected in these final water concentrates (Table1).

Cryptosporidium oocyst detection in mineral waters

In this study, concentrates of mineral water D (with the highest TDS) had no adverse effect on the PCR when compared to the RO water control. As few as 2–5 oocysts were detected by co-amplification with the internal control (Figure 3).

Table 1 Detection of Cryptosporidium species and genotype in UK water samples by PCR-RFLP analysis of filter-extracted DNA

Sample	No. of oocysts $10\ l^{-1}$	IMS volume (ml)	No. of oocysts per filter	Species*	Genotype**
1	NK	18	36	*C. parvum*	1
2	0.07	10	3	*C. parvum*	1
3	0.1	5	12	*C. parvum*	1
4	0.5	5	9	*C. parvum*	1
5	0.9	5	19	*C. parvum*	1
6	0.6	5	27	*C. parvum*	1
7	0.02	5	0	Negative PCR	ND
8	0.015	5	2	Negative PCR	ND
9	0.07	5	2	*C. parvum*	ND

Key: *PCR-RFLP analysis of the 18S rRNA gene with primers CPB-DIAGR/F primers. **Genotyping by PCR-RFLP analysis of the COWP gene (Homan *et al.*, 1999). NK = not known; ND = not done

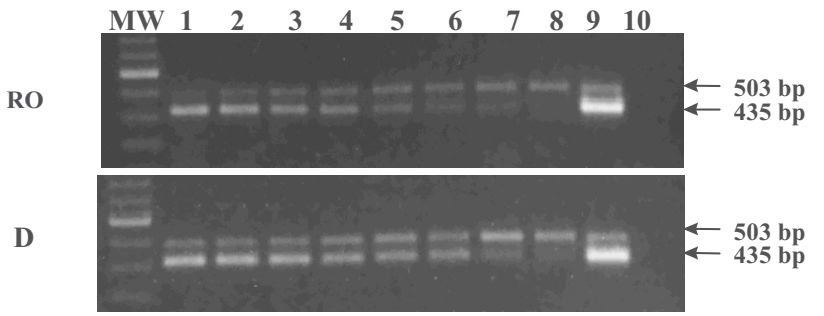

Figure 3 Semi-quantitative detection of small numbers of oocysts seeded in mineral water concentrate

When co-amplification with the competitor was performed, the concentration of the latter was 0.113 attoM (10^{-9} dilution). The amplification of small numbers of oocysts seeded in concentrates of mineral water D, and co-amplified with the competitor, resulted in a PCR positive reaction with DNA equivalent to 5 oocysts detected readily by agarose gel electrophoresis (Figure 3).

Our assay compares favourably with other published PCR-RFLP assays: the primers CPB-DIAGF and CPB-DIAGR are sensitive and genus-specific (Johnson *et al.*, 1995). Developing PCR-RFLP assays for *Cryptosporidium* spp. identification of oocysts found in environmental samples is not without challenges since more than one species may be present in a sample, and the low concentrations of PCR products when amplifying small numbers of oocysts of various species can limit the level of confidence in the assay. The use of different restriction endonucleases can help in species identification.

Lane MW = 100 bp DNA ladder. Lanes 1 to 8 (RO and mineral water D) = PCR product resulting from the co-amplification of approximately 20; 16; 10; 7; 4; 2; 1 and 0.5 oocysts and fixed concentration of competitor (10^{-9} dilution). Lane 9 = positive control. Lane 10 = negative control.

Here we show that as few as 2 oocysts, entrapped on microscope slides or membrane filters, stained by IF and DAPI, when subjected to DNA extraction generated a visible band on agarose gel (Table 1). PCR detection of oocysts following direct DNA extraction from IF and DAPI stained oocysts, entrapped on microscope slides or membrane filters, has not been demonstrated previously. Initial examination by epifluorescence microscopy permits oocyst identification and enumeration by IF and DAPI staining, followed by molecular typing.

Further molecular analysis of the sample can provide information on *Cryptosporidium* species (RFLP-PCR) or *C. parvum* genotype (genotype 1 or 2 by sequencing of PCR product). PCR amplification of the extracted DNA with primers of different specificity can lead to further information regarding host origin and other pertinent information about the parasite including viability, infectivity and virulence, etc. Finally, the internal control safeguards against false negative PCR results and should be particularly useful when drinking water concentrates from varying sources, containing various inhibitors, and new sources of mineral waters are tested.

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