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Identification of *Cryptosporidium* spp. Oocysts in United Kingdom Noncarbonated Natural Mineral Waters and Drinking Waters by Using a Modified Nested PCR-Restriction Fragment Length Polymorphism Assay

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We describe a nested PCR-restriction fragment length polymorphism (RFLP) method for detecting low densities of *Cryptosporidium* spp. oocysts in natural mineral waters and drinking waters. Oocysts were recovered from seeded 1-liter volumes of mineral water by filtration through polycarbonate membranes and from drinking waters by filtration, immunomagnetizable separation, and filter entrapment, followed by direct extraction of DNA. The DNA was released from polycarbonate filter-entrapped oocysts by disruption in lysis buffer by using 15 cycles of freeze-thawing (1 min in liquid nitrogen and 1 min at 65°C), followed by proteinase K digestion. Amplicons were readily detected from two to five intact oocysts on ethidium bromide-stained gels. DNA extracted from *Cryptosporidium parvum* oocysts, *C. muris* (RN 66), *C. baileyi* (Belgium strain, LB 19), human-derived *C. meleagridis*, *C. felis* (DNA from oocysts isolated from a cat), and *C. andersoni* was used to demonstrate species identity by PCR-RFLP after simultaneous digestion with the restriction enzymes *Dra*I and *Vsp*I. Discrimination between *C. andersoni* and *C. muris* isolates was confirmed by a separate, subsequent digestion with *Dde*I. Of 14 drinking water samples tested, 12 were found to be positive by microscopy, 8 were found to be positive by direct PCR, and 14 were found to be positive by using a nested PCR. The *Cryptosporidium* species detected in these finished water samples was *C. parvum* genotype 1. This method consistently and routinely detected >5 oocysts per sample.

Cryptosporidium is a coccidian protozoan parasite that has been implicated in numerous water-borne and food-borne outbreaks of cryptosporidiosis (11, 14, 39). Twelve species of *Cryptosporidium* are considered to be valid and potentially can be found in the environment: *Cryptosporidium hominis* predominantly in humans (30), *C. parvum* in humans and other mammals (46, 48), *C. andersoni* in cattle (27), *C. muris* in mice (47), *C. felis* in cats (2, 19), *C. wrairi* in guinea pigs (7, 50), *C. meleagridis* in turkeys (38), *C. baileyi* in chickens (8), *C. saurophilum* in lizards (23), *C. serpentis* in snakes (26), and *C. nasorum* (18) and *C. molnari* (1) in fish.

Cryptosporidium oocysts are frequent contaminants of water, with contributions from infected human and nonhuman hosts, livestock and agricultural practices and infected feral and transport hosts (40–42). Recent genetic analyses reveal that more than one species of *Cryptosporidium* can infect susceptible, immunocompromised (4, 13, 16, 29, 33, 34, 35), and immunocompetent (21, 33, 34, 53) human hosts, but *C. parvum* remains the most common species infecting humans. *Cryptosporidium* species reported to have crossed host-specificity barriers and to have been detected in human stools include *C. meleagridis*, *C. felis*, *C. muris*, and *C. parvum* dog genotype.

The environmental robustness of oocysts permits prolonged persistence in the aquatic environment (11, 45). Oocysts occur at low densities in water (40–42) and sensitive molecular methods, which can determine species and genotype small numbers of organisms reliably and reproducibly from water concentrates, are required. In particular, sensitivity must be maximized for detecting oocysts in natural mineral water sources since the expected level of contamination should be low because mineral water sources are situated in protected sites and exploited under strict regulations. The risk of contamination can derive from increased exploitation of new sites due to steadily increasing product consumption. In the United Kingdom, bottled water consumption has increased from >5 million liters in 1975 (43) to 1,380 million liters in 2000 (www.globaldrinks.com), and in Western Europe the consumption of mineral waters remains high: 91 liters per annum per capita (www.zenithinternational.com). Protozoan contamination of mineral water was detected in three brands of Mexican bottled mineral waters after membrane filtration and in vitro culture. Rivera et al. (37) identified four types of trophic or cystic forms of parasites, including the nonpathogenic (growth in vitro at 28°C but not at 37°C) amoebae *Vahlkampfia vahlkampfi*, *Naegleria gruberi*, and *Acanthamoeba astronyxis* and one flagellate (*Bodomorpha minima*). In France, a nonpathogenic amoebic species was detected in mineral waters originating from small-capacity, regional plants (9). Recently, *Cryptosporidium* oocysts were detected by immunofluorescence after membrane filtration (oocyst concentration range, 0.2 to 0.5 liter^{-1}) in 2 of 13 commercial brands of Brazilian noncarbonated mineral waters (12).

Environmental contamination with oocysts of *Cryptosporidium* species that are not infectious to susceptible human hosts contributes to the difficulties in assessing the risk to

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public health from water-borne oocysts. The extent of the occurrence of species other than *C. parvum* in the environment is only now being addressed. Xiao et al. (52) reported the analysis of 29 storm water samples in the United States, which revealed the presence of *Cryptosporidium* spp. in 27 of them, mainly wildlife *Cryptosporidium* genotypes. The most common genotypes and species found in surface waters were *C. parvum* genotypes 1 and 2 and *C. andersoni*, with *C. andersoni* reported to be the most commonly found in wastewater (eight samples). However, restriction fragment length polymorphism (RFLP) patterns indicated mixed populations, and sequence analysis of the amplicons indicated that only four genotypes had 100% homology with previously known sequences.

Microscopy is used in both the United Kingdom Regulatory and U.S. standard methods (UK SI no. 1524 and U.S. EPA 1622 and 1623) to identify and differentiate *Cryptosporidium* spp. oocysts by assessing their morphology and morphometry. For many *Cryptosporidium* species present in water concentrates, oocyst size and shape are similar, making species identification based on morphometry difficult, if not impossible, due to size overlap.

Here, we describe a method for the detection and simultaneous species identification of small numbers of *Cryptosporidium* oocysts in United Kingdom finished drinking waters and noncarbonated, natural mineral waters by using epifluorescence microscopy, nested PCR and RFLP with a DNA template prepared directly from oocysts deposited on polycarbonate filters.

MATERIALS AND METHODS

***Cryptosporidium* oocyst sources.** Cervine-ovine oocysts of the Moredun isolate (genotype 2; MD, Moredun Scientific, Ltd., Edinburgh, United Kingdom) and the bovine Iowa isolate (genotype 2; Pleasant Hill Farm [PHF], Troy, Idaho) were obtained commercially. *C. muris* (RN 66) and *C. baileyi* (Belgium strain, LB 19) isolates were donated by K. Webster (Veterinary Laboratories Agency, Weybridge, United Kingdom). Purified human-derived *C. meleagridis* oocysts were obtained from the Scottish Parasite Diagnostic Laboratory *Cryptosporidium* oocyst isolate bank and from the *Cryptosporidium* Reference Unit, Public Health Laboratory Service, Swansea, United Kingdom. Purified DNAs from *C. felis* (oocysts isolated from a cat) and *C. andersoni* (oocysts isolated from a cow) were donated by G. Lindegard, Cornell University.

Mineral waters. Four commercially available, noncarbonated natural mineral waters were selected to include a representative range of total dissolved solids (TDS). These were categorized as mineral waters A (430 mg liter^{-1}), B (280 mg liter^{-1}), C (136 mg liter^{-1}), and D (91 mg liter^{-1}). The TDS values are classified by the Natural Mineral Waters Regulations as follows: very low (<50 mg liter^{-1}), low (50 to 500 mg liter^{-1}), medium (500 to 1,500 mg liter^{-1}), and rich (>1,500 mg liter^{-1}) (5). Mineral waters classified as rich are usually carbonated and were deliberately excluded from the present study. Reverse osmosis (RO) water was used as a reference control. Natural mineral waters were donated by the manufacturers in glass or plastic containers with a 1-, 2.5-, or 5-liter capacity.

Preparation and seeding of mineral water concentrates. The mineral water with the high (430 mg liter^{-1}) TDS left a white, water-soluble salt deposit when filtered through 1.2-μm-pore-size cellulose nitrate membranes, within which seeded oocysts were contained. To simulate this environment for PCR, we filtered 5 liters of mineral waters A, B, C, and D through 1.2-μm-pore-size, 47-mm-diameter cellulose nitrate membranes and dissolved the membrane-entrapped salts in eluant (aqueous solution of 0.1% Tween 80 and 0.001% Antifoam A; Sigma, London, United Kingdom). Filters were processed as follows: filters were placed inside a sterile 50-ml plastic tube containing 10 ml of eluant and vortexed for 2 min. The eluant was transferred to a 15-ml plastic tube, and the filter-elution-vortexing procedure was repeated in the 50-ml tube by using a further 5 ml of eluant. Both eluants were combined and then centrifuged at 1,500 × *g* for 10 min at 4°C, and the eluant was aspirated to leave 5 ml.

Purified *C. parvum* oocysts of bovine origin (Iowa-PHF; genotype 2) were used for seeding the mineral water concentrates. The density of the oocyst stock suspension used for seeding was determined from the arithmetic mean of three separate hemocytometer counts, and a working dilution of 2×10^4 ml^{-1} was prepared in RO water. Five microliters of this working dilution, containing ca. 100 oocysts, was seeded in 85-μl aliquots of each mineral water concentrate, and the suspension was buffered with 10 μl of 10× lysis buffer before DNA extraction by freezing and thawing (see below). The extracted DNA and an internal control (IC) were coamplified with the CPB-DIAG primers in direct PCRs to determine whether any PCR inhibitors were present in the mineral water concentrates (32).

Recovery of oocysts seeded into 1-liter volumes of mineral water A by membrane filtration. For seeding and recovery experiments, fluorescence-activated cell sorting (36) was used to obtain accurate oocyst (MD isolate) seed preparations containing 100 (range, 98 to 101) intact, unstained oocysts in 2 ml of water. From these suspensions 200-μl aliquots, each containing ca. 10 fluorescence-activated cell-sorted oocysts, were seeded into replicates of 1-liter volumes of mineral water A (with the highest TDS). Seeded oocysts were concentrated by filtration through 3-μm-pore-size, 13-mm-diameter polycarbonate filters (Millipore) by using negative pressure. Filter-entrapped oocysts were then fixed, stained, and observed microscopically (see below).

Enhanced morphological detection of oocysts on filters. MD oocysts trapped on polycarbonate filters by filtration and filter controls, prepared by depositing small numbers (ca. 10) of Iowa-PHF oocysts directly on 3-μm-pore-size, 13-mm-diameter polycarbonate filters by negative pressure, were fixed, stained, and observed directly by microscopy.

The filters containing entrapped oocysts were placed onto microscope slides, air dried at room temperature, methanol fixed, and stained with optimally diluted fluorescein isothiocyanate (FITC)-labeled genus-specific anti-*Cryptosporidium* sp. monoclonal antibody (FITC-MAb; Crypt-a-Glo; Waterborne, Inc., New Orleans, La.) according to the manufacturer's instructions. The fluorogenic dye DAPI (4′,6′-diamidino-2-phenylindole) was used to visualize sporozoite nuclei according to the method of Grimason et al. (15). A total of 50 μl of DAPI (final concentration of 4×10^{-4} mg ml^{-1} in 150 mM phosphate-buffered saline [pH 7.2]) was applied onto each sample for 2 min at room temperature. Filters were washed once in RO water to remove excess DAPI, mounted in glycerol–phosphate-buffered saline (60:40 [vol/vol]) containing 2% antifadant 1,4-diazabicyclo(2,2,2)octane (Dabco); a coverslip was then applied, and the filters were examined by epifluorescence microscopy (×400 magnification) on an Olympus BH2 microscope. FITC emissions were viewed by using a blue filter block (480-nm excitation, 520-nm emission), and DAPI emissions were viewed by using a UV filter block (350-nm excitation, 450-nm emission).

DNA extraction from oocysts either in suspension or directly from filters. A freeze-thaw protocol, which maximizes DNA extraction from oocysts, was followed (31; R. A. B. Nichols and H. V. Smith, unpublished data) and applied either to oocysts in suspension or to filter-entrapped oocysts.

Oocysts in lysis buffer (50 mM Tris-HCl [pH 8.5], 1 mM EDTA [pH 8], 0.5% sodium dodecyl sulfate) were subjected to 15 freeze-thaw cycles (1 min in liquid nitrogen and 1 min at 65°C per cycle). Samples were microcentrifuged at 14,000 × *g* for 10 s and then incubated with proteinase K (final concentration, 200 μg ml^{-1}) at 55°C for 3 h, while being rotated horizontally in a Techne hybridization oven. Tubes were then incubated for 20 min in a water bath at 90°C to denature proteinase K, chilled on ice for 1 min, and centrifuged at 14,000 × *g* for 5 min. The supernatant was transferred to a clean tube and either used immediately for PCR amplification or stored at −20°C until used.

Filters were retrieved from slides after microscopic observation by gently immersing the slide vertically in a container of clean RO water to detach the coverslip and then by lifting the filter from the slide by using clean forceps. Each filter was then placed in a 0.5-ml screw-cap microcentrifuge tube and thoroughly wetted in 50 μl of lysis buffer by rolling the capped centrifuge tube between finger and thumb. Oocysts were lysed by using the freeze-thaw protocol described.

PCR protocol. PCR amplifications were performed in a Perkin-Elmer thermocycler model 9600 in 0.5 ml thin-walled tubes. Reaction volumes of either 50 or 100 μl consisted of premixed reagents containing a 200 mM concentration of each of the four deoxynucleoside triphosphates (Pharmacia), 0.2 μM concentrations of each of primers CPB-DIAGF/R (MWG-Biotech, Milton Keynes, United Kingdom), bovine serum albumin at a final concentration of 400 μg ml^{-1}, 3.5 mM $MgCl_2$, 2% Tween 20 (final concentration), and 2.5 U of *Taq* polymerase in 1× PCR buffer IV (Advanced Biotechnologies). DNA template did not exceed 10% of the reaction volume.

Nested PCR assay. To develop a nested PCR assay according to established principles (10, 17), a primer set, which flanked the amplicon defined by the CPB-DIAGF/R primers, was designed. The outer primers were designed to have an annealing temperature significantly higher than the inner primers so that a single-tube nested PCR could also be developed, if required. The GC content

TABLE 1. *Cryptosporidium* spp. and genotypes determined by RFLP of the amplicon defined by the CPB-DIAGR/F primers after digestion with the enzymes *Vsp*I, *Dra*I, and *Dde*I

Cryptosporidium spp. and genotypes (total amplicon length in bp)	Amplicon length(s) (bp) as determined with:			GenBank accession no.
	*Vsp*I	*Dra*I	*Dde*I	
C. parvum 1 (438)	222, 104, 112	None	204, 68, 166	L16997
C. parvum 2 (435)	219, 104, 112	None	201, 68, 166	L16996, AF161856
C. muris (432)	320, 112	None	42, 224, 166	AF093498, AF093498, AF093497
C. andersoni (431)	319, 112	None	265, 166	AF093496, L19069
C. felis (455)	239, 104, 112	50, 405	221, 68, 166	AF087577
C. baileyi (428)	212, 104, 112	84, 344	262, 166	L19068, AF093495
C. meleagridis (434)	47, 171, 104, 112	None	200, 68, 166	AF112574
C. serpentis (430)	318, 112	None	264, 166	AF093502[a]
C. wrairi (435)	219, 104, 112	None	201, 68, 166	AF115378
Cryptosporidium pig (435)	219, 104, 112	None	201, 68, 166	AF108861
Cryptosporidium desert monitor (432)	216, 108, 112	None	198, 68, 166	AF112573
Cryptosporidium mouse (439)	48, 175, 104, 112	None	205, 68, 166	AF108863
Cryptosporidium ferret (438)	48, 174, 103, 113	None	204, 68, 166	AF112572
Cryptosporidium dog (429)	213, 104, 112	None	195, 68, 166	AF112576
Cryptosporidium koala (436)	220, 104, 112	None	202, 68, 166	AF108860
Cryptosporidium kangaroo (436)	220, 104, 112	None	202, 68, 166	AF112570
Cryptosporidium monkey (436)	220, 104, 112	None	202, 68, 166	AF112569
Cryptosporidium bear (432)	216, 104, 112	None	87, 111, 68, 166	AF247535

[a] Also AF151376, AF093500, and AF093501.

was 59.2 and 53.8% forward and reverse primers, respectively, and care was taken to choose a noncomplementary primer set, particularly at the 3′ ends of the two primers, to avoid primer-dimer formation.

A 27-mer forward primer (N-DIAGF2; 5′-CAA TTG GAG GGC AAG TCT GGT GCC AGC-3′) and a 26-mer reverse primer (N-DIAGR2; 5′-CCT TCC TAT GTC TGG ACC TGG TGA GT-3′) were selected as they had homology with all *Cryptosporidium* species and genotypes listed in Table 1. The expected lengths of amplicons obtained after amplification with N-DIAGF2/R2 vary from 655 to 667 bp depending on the species of *Cryptosporidium* or *C. parvum* genotype.

Optimum annealing temperatures for both primer sets (CPB-DIAGF/R and N-DIAGF2/R2) were determined by PCR amplification of DNA equivalent to two oocysts, obtained by serial dilution of DNA extracted from 10^6 oocysts (Iowa isolate, genotype 2; extracted as described previously). Both outer and inner primers were evaluated by direct PCR assays at the optimum concentration of 200 nM each per reaction tube. The primary and secondary PCR reagent concentrations were as described above. The primary PCR contained 2 μl of DNA in a 50-μl reaction volume, and the secondary PCR reamplified 5 μl of the primary PCR product. The following step cycle protocols were used: primary PCR consisted of initial denaturation at 95°C for 5 min; 25 to 35 cycles at 94°C for 30 s, 68°C for 1 min, and 72°C for 30 s; and extension at 72°C for 10 min. Secondary PCR consisted of initial denaturation at 95°C for 1 min; 25 to 35 cycles at 94°C for 30 s, 60°C for 1 min, and 72°C for 30 s; and extension at 72°C for 10 min.

To minimize cross-contamination pre-PCR, DNA extraction and template addition to reaction tubes and post-PCR manipulations were performed in three separate laboratory areas by using designated micropipettes and filtered tips.

***Cryptosporidium* oocyst detection in United Kingdom drinking water samples.** Fourteen United Kingdom finished water samples were concentrated and oocysts were purified by immunomagnetizable separation (IMS) according to United Kingdom Standard Operating Protocols identified in part 2 of the protocol for monitoring *Cryptosporidium* oocysts in water supplies (49) generated to implement the Water Supply (Water Quality) (Amendment) Regulations of 1999 (SI no. 1524). IMS separations from turbid samples were performed in subsamples whenever necessary to have packed pellet volumes equal to 0.5 ml of sediment in a 10-ml sample volume (5% packed pellet volumes). Oocysts recovered from the IMS subsamples were pooled on one polycarbonate membrane filter (13-mm diameter and 1.2-μm pore size) for epifluorescence microscopy and subsequent DNA extraction. The finished water concentrates were analyzed for the presence of *Cryptosporidium* oocysts by using epifluorescence microscopy, direct PCR, and nested PCR.

Species identification by PCR-RFLP analysis. Purified oocysts of *C. parvum* (genotype 2; human isolate), *C. muris*, *C. baileyi*, and *C. meleagridis* and DNA from *C. felis* and *C. andersoni* oocysts were used to confirm species identity by PCR-RFLP. Approximately 10^3 oocysts of each species suspended in 100 μl of PCR buffer were subjected to DNA extraction by freeze-thawing, and 20 μl of the supernatant was used for PCR amplification. Then, 10 μl of the PCR product was digested for 2 h at 37°C with 10 U of each enzyme *Dra*I and *Vsp*I (Gibco/Life Technologies) in 35 μl of 1× React-2 buffer (Gibco/Life Technologies/Invitrogen). Undigested controls were run alongside the digested fragments in 2% agarose gels at 100 V for 2 h and then stained with ethidium bromide (0.5 μg ml^{-1}) incorporated into both gel and running buffer. The interpretation of nested PCR-RFLP results was performed as described in Table 1.

RESULTS

Direct PCR detection of oocysts seeded into mock concentrates of natural mineral waters. Direct PCR coamplification of DNA extracted from 100 oocysts seeded in concentrated mineral waters A, B, C, and D and the IC yielded two bands; a 435-bp band corresponding to amplicons generated by the amplification of 10 oocysts, and a 503-bp band corresponding to the amplification of the IC. No inhibitory effect on the PCR occurred when the mineral waters concentrates were tested (data not shown).

Direct PCR and nested PCR detection of oocysts experimentally entrapped on membrane filters by filtration of seeded United Kingdom mineral water A. Enumerations of oocysts recovered from replicate filtrations of mineral water A and filter controls by microscopy are presented in Table 2. Direct PCR coamplification of 10 μl of DNA template extracted from each membrane containing entrapped, methanol-fixed, FITC-antibody, and DAPI-labeled oocysts with 10 μl of the IC yielded a product corresponding to the IC (553 bp) from all mineral water A replicate samples tested (data not shown). However, genomic DNA (435 bp) amplicons either were generated at low levels or were undetectable on an agarose gel (Table 2).

Using the nested PCR assay with DNA from filter-entrapped oocysts by mineral water A filtrations generated detectable amplicons when 50 cycles of PCR amplification were performed. The sensitivity of the nested PCR assay was two to three oocysts present on each filter with PCR product detected

TABLE 2. Comparison of the sensitivity of epifluorescence microscopy, direct PCR, and nested PCR for detecting oocysts retained on membrane filters

Filter	No. of oocysts counted (FITC labeled/DAPI labeled[a])	Sensitivity[b] as determined by:	
		Direct PCR	Two-step nested PCR
F filters[c]			
F1	11/6	Negative	ND
F2	6/5	1+	4+
F3	4/0	1+	4+
F4	8/7	Negative	4+
F5	7/4	Negative	4+
F6	8/4	Negative	3+
F7	7/5	Negative	4+
F8	4/2	Negative	2+
F9	6/5	Negative	1+
F10	12/10	Negative	3+
F11	10/7	1+	4+
F12[e]	9/2	Negative	4+
FC filters[d]			
FC1	5/2	Negative	3+
FC2	9/2	Negative	4+
FC3	9/3	Negative	4+
FC4	4/1	Negative	4+
FC5	15/9	Negative	3+
FC6	2/1	1+	3+
FC7	13/10	1+	4+
FC8	7/2	Negative	3+
FC9	12/5	Negative	3+

[a] DAPI labelling refers to oocysts containing one to four nuclei.
[b] Negative, no PCR product detected on gel; 4+, PCR product yielding a strong band; 3+ and 2+, PCR product yielding medium-strength bands; 1+, PCR product yielding a weak band on the gel; ND, not done.
[c] F filters, filters through which seeded mineral water A was passed.
[d] FC filters, filter controls (filters onto which a known number of oocysts were deposited [see text]).
[e] Filtration of 1 liter of RO water.

on the gel in sufficient concentration for RFLP analysis (Table 2). No PCR product was ever observed with negative controls.

Detection and specifies identification of *Cryptosporidium* oocysts concentrated from United Kingdom finished drinking waters. (i) Confirmation of species identification by RFLP. The amplification of *C. parvum* genotype 2, *C. muris*, *C. baileyi*, *C. felis*, and *C. meleagridis* oocyst DNA yielded products of 435, 431, 428, 455, and 434 bp, respectively (Table 1 and Fig. 1). On 2% ethidium bromide-stained gels, the double-digest product (with *Dra*I and *Vsp*I) produced profiles that confirmed the reported sequence of these cloned genes (Table 1 and Fig. 1). RFLP analysis of the amplicon defined by the diagnostic primers with restriction enzymes used for identifying *Cryptosporidium* species and *C. parvum* genotypes is presented in Table 1.

(ii) Immunofluorescence, direct PCR, and nested PCR detection of oocysts in finished drinking waters. Oocysts (range, 2 to 37) were detected in 12 of 14 water samples by microscopy on filters (Table 3). Of these, the number of oocysts that contained nuclei (one to four DAPI-stained nuclei per oocyst) ranged from 2 to 30. No oocysts were visible on filters 8 and 11.

Of the 14 DNA samples that were amplified by direct PCR, 8 were found to be positive when 10 μl of lysate was amplified (Table 3). No inhibition of test sample occurred when 5 μl of lysate was coamplified with 5 μl of the IC (10^{-9} dilution), since IC amplicons were observed with all samples (data not shown). A total of 11 samples were determined to be positive (10 strong and 1 weak, sample 13 [1418]) with the nested PCR assay after 50 cycles of nested amplification (samples 11, 12, and 13, Table 3). Increasing the number of amplification cycles to 70 (35 cycles for both the primary and secondary PCRs) yielded amplicons from the three previously negative samples (Table 3). Samples 11 and 12 generated amplicons that were longer (ca. 450 bp) than expected for *C. parvum* (435 bp), and less PCR product was detected visually compared to samples 13 and 14 (data not shown).

(iii) Species and genotype identification of oocysts recovered from finished drinking waters. RFLP analysis of the amplicons by simultaneous digestion with the enzymes *Vsp*I and *Dra*I revealed a profile compatible with *C. parvum* species in 12 of 14 samples (Table 3). Samples 11 and 12 gave identical profiles; these profiles differed from those expected with known species or genotypes in that three fragments were obtained corresponding to ca. 240, 104, and 50 bp. In an attempt to clarify the digestion pattern of these samples, separate restrictions with *Dra*I and *Ssp*I were performed, but no digestion product was generated with these enzymes (data not shown). Of the drinking water concentrates tested, six were genotyped with the single-tube nested COWP assay (17), and RFLP anal-

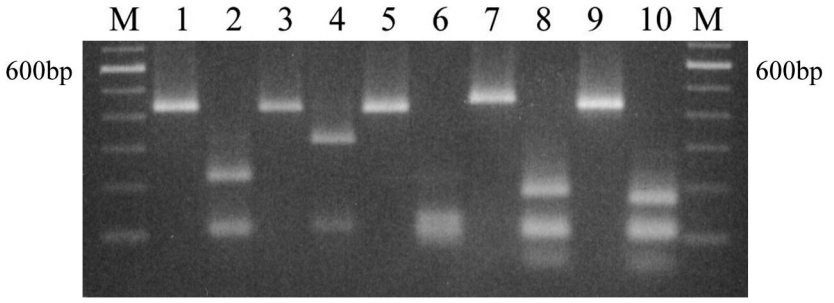

FIG. 1. Two percent agarose gel electrophoresis analysis of amplicons before and after simultaneous digestion with restriction enzymes *Dra*I and *Vsp*I. Lanes 1, 3, 5, 7, and 9, undigested PCR product from *C. parvum*, *C. muris*, *C. baileyi*, *C. felis*, and *C. meleagridis*, respectively; lanes 2, 4, 6, 8, and 10, fragmented PCR product from *C. parvum* (222, 112, and 104 bp), *C. muris* (319 and 112 bp), *C. baileyi* (128, 112, 104, and 84 bp), *C. felis* (189, 112, 104, and 50 bp), and *C. meleagridis* (171, 112, 104, and 47 bp), respectively. Lanes M, 100-bp DNA ladder. In this figure, bands of sizes 112 and 104 bp comigrate and appear as a single band. This does not affect the interpretation of the RFLP analysis nor the discrimination among the five species.

TABLE 3. Detection of *Cryptosporidium* species and *C. parvum* genotype by nested PCR-RFLP analysis of filter-extracted oocyst DNA in United Kingdom finished water samples

Sample (no.)	No. of oocysts 10 liters^{-1}	IMS vol (ml)	Total no. of labeled oocysts (FITC/DAPI)	Result[a] as determined by:		RFLP sp.[b]
				Direct PCR	Nested PCR	
1 (1285)	NK[c]	5	4/4	2+	4+	*C. parvum*
2 (1285)	NK	18	37/30	2+	4+	*C. parvum*†
3 (1304)	0.07	10	3/3	1+	4+	*C. parvum*†
4 (1305)	0.1	5	12/10	2+	4+	*C. parvum*†
5 (1291)	0.5	5	10/8	2+	4+	*C. parvum*†
6 (1293)	0.9	5	19/15	2+	4+	*C. parvum*†
7 (1297)	0.6	5	27/24	1+	4+	*C. parvum*†
8 (1315)	0.02	5	0/0	Negative	4+	*C. parvum*
9 (1372)	0.015	5	2/2	Negative	4+	*C. parvum*
10 (1389)	0.07	5	2/2	1+	4+	*C. parvum*
11 (1468)	0.02	5	0/0	Negative	2+*	NI
12 (1419)	0.05	5	2/1	Negative	2+*	NI
13 (1418)	0.09	5	5/5	Negative	4+*	*C. parvum*
14 (1416)	0.1	5	7/6	Negative	4+	*C. parvum*

[a] Amplicons detected after 70 cycles of amplification are indicated by an asterisk. 4+, 3+, 2+, and 1+ are as described in Table 2, footnote *b*.
[b] A dagger shows *C. parvum* genotype 1 (single-tube nested COWP PCR-RFLP assay [17]). NI, not identified.
[c] NK, not known.

ysis of the PCR product indicated that *C. parvum* genotype 1 was the *Cryptosporidium* genotype detected (Table 3).

DISCUSSION

Both species identification and detection of small numbers of oocysts are prerequisites for a successful PCR-based method for detecting *Cryptosporidium* oocysts in natural mineral waters and in drinking waters. PCR amplification of multicopy genes is a useful approach to sensitive molecular detection. Le Blancq et al. (24) determined that 20 copies of the *Cryptosporidium* ribosomal DNA gene are present per oocyst. Since the complete sequence of the small-subunit (SSU) rRNA gene from oocysts originating from numerous hosts and from different *Cryptosporidium* species is currently available from the GenBank database, species identification by PCR-RFLP analysis of this *Cryptosporidium* gene has been used by many authors (3, 22, 25, 28, 51).

Leng et al. (25) devised primers that amplify the entire 18S rRNA gene based on sequences deposited in the GenBank by Pieniazek et al. (N. J. Pieniazek, M. J. Arrowood, B. L. Blagburn, H. M. Mathews, and S. B. Slemenda, unpublished data [GenBank accession no. L16996]) and confirmed the feasibility of distinguishing among *C. parvum*, *C. muris*, and *C. baileyi* by PCR-RFLP by double digestion with the enzymes *Dra*I and *Vsp*I. These primers, aimed at conserved sequences of the gene, also amplify *Eimeria neischultzi* and *E. papillata* DNA (44). In contrast, the diagnostic primers used in the present study are suitable for species identification by PCR-RFLP (Table 1). To our knowledge this is the first report to confirm the GenBank sequences by simultaneous restriction digestion with the enzymes *Dra*I and *Vsp*I using PCR products obtained with the diagnostic primers CPB-DIAGF and CPB-DIGR of Johnson et al. (20).

Champliaud et al. (6) compared the performance of eight primer pairs described in the literature for their ability to distinguish among *C. parvum*, *C. muris*, *C. baileyi*, and *C. meleagridis*. These authors showed that all primer pairs amplified DNA from *C. meleagridis* and *C. parvum*, including the two genus-specific primer pairs directed to the 18S rRNA gene (3, 20) that amplified DNA from all four species. Digestion of the 18S rRNA gene product, obtained with the primers developed by Awad-El-Kariem et al. (3), with *Mae*I yielded incomplete digestion, as expected. However, these authors did not digest the PCR products obtained with the primers of Johnson et al. (20) with the enzymes *Dra*I and *Vsp*I used in the present study.

Complete sequences of the SSU rRNA gene from different isolates of *C. parvum*, *C. muris*, *C. baileyi*, and *C. serpentis* have been deposited in GenBank (51). A phylogenetic study by sequence analysis of this gene locus confirmed these species as distinct taxonomic groups. A nested PCR-RFLP assay for interspecies discrimination was devised by digesting the PCR product with *Ssp*I, and intraspecies variation of *C. parvum* from human or animal genotypes was tested by digestion with *Vsp*I (51). The sensitivity of this test with purified oocysts was reported to be of a single oocyst; however, the sensitivity and specificity of detection in environmental samples are yet to be evaluated fully.

In the present study we have shown that species identification can be accomplished by nested PCR-RFLP from the amplification of five or fewer oocysts entrapped on polycarbonate membranes and isolated from noncarbonated, natural mineral waters and from drinking waters. When optimized PCR and DNA extraction protocols are used, these primers are sensitive and suitable for use in direct PCR and nested PCR assays. The five *Cryptosporidium* species simultaneously detected because of the polymorphism of this region of the 18S rRNA, defined by the CPB-DIAGF and CPB-DIGR primers, are relevant to a detection method for natural mineral and drinking waters since these species can be present in the United Kingdom environment. Differentiation between *C. andersoni* and *C. muris* is possible by further digesting the PCR product with the restriction enzyme *Dde*I (data not shown).

In addition, filter concentrates from noncarbonated mineral water do not interfere with the PCR and the DNA extracted from methanol-fixed and FITC-MAb and DAPI-stained oocysts entrapped on polycarbonate membranes does not inter-

fere with nested PCR amplification, given the current sensitivity of two to five oocysts. Since only 20% of the extracted DNA is used for the first amplification, other PCR tests, including *C. parvum* genotyping, can also be performed on the same sample by amplifying other polymorphic loci with sensitive and specific primers (17).

The identification of *C. parvum* genotype 1 (renamed *C. hominis* [30]) DNA in all finished drinking water concentrates is compatible with the fact that the water samples were collected from an oocyst-contaminated drinking water strongly associated with a *C. parvum* genotype 1 outbreak. Thus, our nested PCR appears to be more sensitive than microscopy for detecting oocysts in these water concentrates. In concentrates that had no identifiable oocysts present, occluding debris may have obscured oocyst observation by microscopy; however, given that the water samples were collected from a *Cryptosporidium*-positive water source, the presence of amplifiable, naked DNA in the water concentrate remains a plausible explanation for these results. We identified the same *Cryptosporidium* species and *C. parvum* genotype both in outbreak-associated human stools and in the oocysts present in the finished drinking water supplying that outbreak area. Sequencing the PCR products obtained from samples 11 and 12 may assist in elucidating the *Cryptosporidium* species-*C. parvum* genotype signature of the amplifiable *Cryptosporidium* DNA isolated.

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