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Extraction and Genotyping of *Cryptosporidium parvum* DNA from Fecal Smears on Glass Slides Stained Conventionally for Direct Microscope Examination

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A method was developed for extracting cryptosporidial DNA from stained fecal smears on glass microscope slides. The correct genotype of *Cryptosporidium parvum* was amplified by PCR from 89 (85%) of 105 smears following conventional staining but not from negative controls. This technique may have applications for analysis of other infectious agents.

Cryptosporidium parvum is an intestinal protozoan that infects both humans and a wide range of animals, including livestock (5). In humans, the organism causes a self-limiting enteritis in immunocompetent individuals but can be life threatening when immunocompromised subjects are infected (5). The diagnosis of cryptosporidiosis principally relies on the recognition of cryptosporidial oocysts by light microscopy in stained fecal smears; diagnostic staining techniques include immunofluorescence (IF), modified Ziehl-Neelsen (MZN), and auramine phenol (AP) methods (2).

Genetic analysis of a large number of independent loci of *C. parvum* indicates that this parasite comprises at least two genotypes: genotype 1 is exclusive to naturally infected humans and a nonhuman primate, and genotype 2 infects a much broader host range, including humans and livestock (7). One genetic locus which allows differentiation between the two genotypes of *C. parvum* is the *Cryptosporidium* oocyst wall protein (COWP) gene, and genotyping methods based on PCR/restriction fragment length polymorphism (RFLP) analysis of a fragment of this gene have been described (8, 9).

C. parvum genotyping techniques using various polymorphic loci have been most often applied to DNA extracted from purified oocyst suspensions (10) or from whole feces containing oocysts (6). However, since diagnosis relies on the recognition of oocysts in stained fecal smears and since all samples from patients with diarrhea are examined by microscopy for *Cryptosporidium* (at least within diagnostic laboratories of the Public Health Laboratory Service in England and Wales) (1), stained microscope slides with fecal smears represent a further potential source of parasite material previously unused for secondary testing such as genotyping.

The purpose of this study was to establish if successful extraction and genotyping of cryptosporidial DNA could be achieved from fecal smears on glass microscope slides stained by conventional procedures.

Fecal samples were collected from patients with diarrhea in the United Kingdom during 1998 and 1999; using conventional techniques, *Cryptosporidium* (oocysts), *Cyclospora* (oocysts), *Giardia* (cysts), and *Clostridium perfringens* (bacteria and enterotoxin) were detected or no known etiological agent was detected. All fecal samples were stored at +4°C without preservatives for up to 2 years.

Fecal smears were prepared by air drying 30 μl of homogenized stool sample spread in an even smear over approximately 75% of the area of a glass microscope slide. The smears were fixed with either acetone or methanol and stained for cryptosporidial oocysts by the IF, AP, or MZN method as described elsewhere (2, 4, 6). Numbers of oocysts were counted, and an estimate of the levels was calculated from the mean of 20 fields using a 40× objective (Zeiss, Welwyn Garden City, United Kingdom).

Oocyst disruption and DNA purification from whole feces were performed as described elsewhere (6).

Stained fecal smears on microscope slides were stored at room temperature, and DNA was extracted within 2 weeks of preparation. To extract DNA, the stained slide was placed into a 50-ml conical tube (Falcon) with 900 μl of L6 buffer (10 M guanidinium thiocyanate in 0.1 M Tris HCl [pH 6.4]–0.2 M EDTA [pH 8.0]–2% [wt/vol] Triton X-100 [3]). Material was removed from the slide by vigorously rubbing the stained surface for 30 s with a sterile cotton swab (Medical Wire and Equipment Co., Corsham, Wiltshire, United Kingdom). The head of the swab was then removed and placed in a 2-ml microcentrifuge tube (Sarstedt) containing 0.3 g of 0.5-mm-diameter zirconia beads (Stratech Scientific, Luton, United Kingdom). The conical tube containing the slide was centrifuged for 5 min at 1,000 × *g*, the glass slide was discarded, and 60 μl of isoamyl alcohol was added. All material was transferred to the microcentrifuge tube containing the swab head and zirconia beads, which was then disrupted by shaking in a Beatbeater-8 (Stratech Scientific) for 1.5 min at maximum speed. Following centrifugation of the mixture, the particulate material was discarded and 100 μl of activated silica (Severn

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TABLE 1. Results of *C. parvum* COWP gene amplification using DNA recovered from stained microscope slides and whole feces

Genotype detected in whole feces[a]	No. of slides	Extraction of DNA from stained slides[b]: no. (%) of samples with COWP gene amplification	
		Genotype 1	Genotype 2
1	54	43 (80)	0
2	51	0	46 (90)
Total	105	89 (85)	

[a] Unnested PCR procedure.
[b] Twenty of which were stained by the IF method, 60 by the MZN method, and 25 by the AP method; PCR using nested procedure.

Biotech) was added to the supernatant. DNA was purified by washing the silica twice with L2 buffer (10 M guanidinium thiocyanate in 0.1 M Tris-HCl [pH 6.4]), twice with ice-cold 80% ethanol, and once with ice-cold acetone (3, 6). The silica was dried (55°C for 10 min), 150 μl of water was added, the mixture was reincubated at 55°C for 5 min, and the supernatant (DNA sample) was recovered by centrifugation.

Genotyping of *C. parvum* present in all whole feces was achieved using an unnested amplification of the COWP gene fragment followed by RFLP analysis as described elsewhere (9). Analysis of DNA extracted from microscope slides was performed using either a nested (8) or an unnested (9) COWP gene fragment amplification followed by RFLP analysis as above. Positive (previously tested extracts from whole feces generating fragments of known genotype) and negative (buffer only) controls were included in each experiment, and amplified product was detected in 1% agarose electrophoresis gels. The cryptosporidial genotype was determined by analysis of *Rsa*I digestion fragments in 3.2% agarose gels. All gels were stained with ethidium bromide and recorded under UV transillumination using type 667 film (Polaroid Ltd., St. Albans, United Kingdom).

Initial experiments using DNA extracted from stained fecal smears on microscope slides where *C. parvum* oocysts had been detected by microscopy showed that the unnested COWP PCR procedure (9) was insufficiently sensitive to amplify DNA extracted from the majority of slides tested. However, with the nested procedure (8), the COWP gene fragment was amplified from 89 (85%) of 105 stained fecal smears positive for *C. parvum* oocysts (Table 1), 20 of which were stained by the IF method, 60 by the MZN method, and 25 by the AP method. There were no significant differences between the proportions of slides where DNA amplification was achieved following staining by each of the three different methods (IF, MZN, or AP). Of the 89 slides where amplification was achieved, identical genotyping results were obtained with DNA extracted from stained fecal smears and from whole feces (Table 1). Triplicate smears were prepared from 22 samples of feces, and these were stained by all three procedures. DNA was extracted from all 66 slides, and identical COWP amplification results were obtained from 18 of the triplicates. In the remaining four samples, the COWP gene was amplified from either one or two of the three slides; all of these contained low numbers of oocysts (<1 per field) (see results presented below).

The relationship between numbers of *C. parvum* oocysts observed by microscopy and the proportion of samples where the COWP gene fragment was amplified for 89 slides is shown in Table 2. One hundred percent amplification of the COWP gene was achieved for DNA extracted from slides that contained ≥1 oocyst per microscope field (approximately equivalent to ≥10,000 oocysts/ml of feces). However, where the number of oocysts was less than one per field, DNA amplification efficiency was 67, 56, and 67% with IF-, MZN-, and AP-stained slides, respectively (Table 2). Hence, the proportion of amplification was dependent upon the absolute numbers of oocysts present.

To investigate the possibility of inhibition of the PCR by material extracted from cotton swabs, dilutions of DNA extracted from whole feces containing *C. parvum* DNA where the COWP gene fragment could be amplified were added to four extracts from slides which contained low numbers of oocysts but where the COWP gene fragment was not amplified. DNA was amplified at similar intensities from all four spiked samples and a spiked water-only control. Furthermore, there were no differences between the highest dilutions where DNA was amplified from either of the four spiked samples or the spiked water-only control. This suggests that there was no significant effect of inhibition of the PCR by material from the cotton swab.

The specificity of the procedure was assessed by extracting DNA from 33 slides stained by IF (11), MZN (11), or AP (11) that had been prepared from feces where *C. perfringens* (6 samples), *Cyclospora* (6 samples), *Giardia* (9 samples), or no etiological agent (12 samples) had been detected. These slides were prepared and stained together with three samples with high numbers of *C. parvum* oocysts already identified as strongly positive for COWP amplification. No false-positive COWP gene amplification was detected.

In summary, *C. parvum* DNA present in fecal smears on microscope slides stained using conventional procedures can be extracted using a simple, albeit destructive, procedure. There is sufficient DNA for genetic characterization of the parasite. No evidence for experimental cross-contamination between slides was obtained from the results of negative and positive controls, the *C. parvum* genotypes obtained from different DNA preparations derived from the same original stool samples, and parallel testing of strongly positive slides together with negative slides. This PCR method is specific for *Cryptosporidium* and is reproducible; however, it is less sensitive than extracting DNA from whole feces (at least a 10-fold reduction in the amount of material is examined compared to that in the method for whole feces [6]), which may be problematic when examining samples with low numbers of oocysts. Further work is in progress to increase the sensitivity of the technique and to assess the stability of cryptosporidial DNA on microscope slides over extended periods. We have already established in

TABLE 2. Effect of *C. parvum* oocyst count on percentage of slides with COWP gene amplification

Staining method	No. of slides	% Amplification of COWP gene relative to no. of oocysts detected/microscope field		
		<1	1–10	>10
IF	20	67	100	100
MZN	60	56	100	100
AP	25	67	100	100

subsequent experiments that DNA can be extracted and satisfactorily amplified from slides stored at room temperature without immersion oil for >2 years and that similar techniques can be used to extract and amplify bacterial DNA from Gram-stained smears (unpublished data). Because of the fixed nature of the fecal smear (reflecting the content of the feces at the time of preparation and not after manipulation), together with advantages in availability and transport, this approach for recovering DNA from stained smears on microscope slides may be applicable to analysis of a wide range of other potential infectious agents.

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