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Detection of *Cryptosporidium parvum* DNA in Formed Human Feces by a Sensitive PCR-Based Assay Including Uracil-*N*-Glycosylase Inactivation

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We developed a PCR-based method that can be used to identify *Cryptosporidium parvum* in human feces. Fecal oocysts were concentrated by centrifugation on a sodium chloride gradient and filtration on a nitrocellulose filter prior to DNA extraction and PCR amplification of a 452-bp *C. parvum*-specific DNA sequence with a protocol including dUTP and uracil-*N*-glycosylase. All samples obtained from naturally infected humans ($n = 10$), calves ($n = 4$), and goats ($n = 2$) were positive. A 100% detection rate was achieved with both formed and solid stools ($n = 10$) seeded with 1,000 *C. parvum* oocysts per g. Procedures based on stool concentration by a modified Ritchie method and subsequent oocyst identification by immunofluorescent labeling or acid-fast staining require concentrations of 50,000 to 500,000 oocysts per g to achieve a 100% detection rate with formed stools. The described PCR-based assay thus has a 50- to 500-fold increase in sensitivity compared to those of the methods commonly used to analyze formed feces.

Cryptosporidium parvum (Protozoa, Apicomplexa) causes worldwide diarrheal disease in humans and young livestock (6, 9). Whereas cryptosporidiosis is self-limited in healthy individuals, AIDS patients generally experience a prolonged life-threatening diarrhea. It has been reported that paromomycin, a nondiffusible oral aminoglycoside, improves the symptoms of AIDS cryptosporidiosis (3, 8, 10, 22, 27), but a high percentage of relapse occurs when the treatment is discontinued (3, 8, 10, 22). Dissemination of the parasite to the biliary mucosa, (9, 25, 27) and the residual parasites at various stages that remain inside intestinal crypts (24) may serve as reservoirs of parasites, responsible for reinfections after a primary paromomycin treatment, therefore explaining the difficulty in achieving a complete parasitologic cure in immunocompromised patients (23, 24). Laboratory diagnosis commonly relies on the recognition of the oocysts in stool specimens after staining with either acid-fast stains or immunofluorescent antibodies. However, the threshold of detection in formed feces may be as high as 50,000 to 500,000 oocysts per g by these methods (26). This poor sensitivity, together with a reduced oocyst excretion in nondiarrheic patients (7, 11, 20), probably accounts for the fact that the diagnosis of cryptosporidiosis is generally made with diarrheic stools from patients excreting high numbers of oocysts, when parasite dissemination is likely to defeat therapeutic efforts (23, 24). In order to improve treatment efficacy, the diagnosis should be established at a less advanced stage of the disease (22). The often progressive nature of cryptosporidiosis in AIDS patients (7, 23, 24) and the possible occurrence of an asymptomatic carrier state (13, 28) may provide opportunities to do so. DNA amplification by PCR is a potentially powerful method of achieving this aim. Three steps are critical in the development of a PCR method aimed at identifying *C. parvum* in stools from human immunodeficiency virus (HIV)-infected patients: sterilization of the specimens to protect laboratory personnel from HIV contamination, efficient recovery of the oocysts to ensure sensitive diagnosis, and incorporation of a protocol to prevent carryover contaminations. We describe herein a PCR method that meets these requirements and that identifies *C. parvum* in both formed and solid feces containing 100 to 1,000 oocysts per g, a level of sensitivity that paves the way to a new approach in the management of cryptosporidiosis in AIDS patients.

All the fecal samples were tested for *C. parvum* oocysts as follows. One drop of the fecal material diluted in physiological saline was spread thinly on a slide, fixed with methanol, and stained by the modified Ziehl-Neelsen technique described by Henriksen and Pohlenz (12) with a 60-min staining in fuchsin. For each sample analyzed, two smears were entirely scanned at a magnification of ×200 by two different investigators, and oocyst identification was carried out at a magnification of ×1,000. Altogether, five groups of specimens were analyzed. Group 1 samples were obtained from 10 HIV-negative patients and consisted of both formed and solid stools which tested negative for *C. parvum* by Ziehl-Neelsen staining. These samples were seeded with purified oocysts (4) of *C. parvum* to yield concentrations of 100 and 1,000 oocysts per g. Group 2 consisted of 16 stool samples which tested negative for *C. parvum* by Ziehl-Neelsen staining and were analyzed without the addition of oocysts. Groups 3, 4, and 5 consisted of stool samples

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which tested positive for *C. parvum* by Ziehl-Neelsen staining and were obtained from naturally infected humans (10 samples; group 3), calves (4 samples; group 4), and goats (2 samples; group 5). The specimens from groups 1 and 2 were stored at 4°C for up to 7 days before use. The specimens from groups 3, 4, and 5 were stored in 2.5% potassium dichromate at 4°C and were washed thoroughly to remove any remaining potassium dichromate before analysis. Treatment for DNA extraction was performed as follows. One-gram portions of fecal material were mixed with 40 ml of 0.35% sodium hypochlorite and were sieved through four layers of wet gauze. Twenty milliliters of the filtrate was mixed with 5 ml of ether and then shaken for 30 s and centrifuged at 1,600 × *g* for 15 min. The pellet, resuspended in 15 ml of deionized water, was layered over a two-layer sodium chloride gradient (specific gravities, 1.1 and 1.05) in 50-ml conical centrifuge tubes. After centrifugation for 12 min at 350 × *g*, 10 ml was recovered at the 1.1–1.05 interface and was diluted with 10 ml of deionized water, and half of this suspension was filtered through 3-μm-pore-size, 25-mm-diameter nitrocellulose filters (Sartorius) which were housed in polycarbonate filter holders. The filters were rinsed with deionized water, then inverted into the filter holder, and finally washed with 3 ml of 10 mM Tris HCl (pH 8) in a 15-ml conical centrifuge tube. Recovery of the oocysts was completed by shaking the filters for 30 s in the corresponding tubes. After discarding the filters, the samples were centrifuged at 16,000 × *g* for 5 min in 1.5-ml conical centrifuge tubes, and the final pellets which were recovered in 50 μl of 10 mM Tris HCl (pH 8) were treated for DNA extraction as described previously (5).

The target of amplification was a 452-bp DNA sequence specific to the *C. parvum* genome (14, 15). To avoid contamination by the amplification products, deoxyuridine triphosphate nucleotides were substituted for deoxythymidine triphosphate nucleotides in the amplification reaction mixture (19). For each sample analyzed, 10 μl of lysate was used as a template in 50-μl reaction mixtures containing 75 mM Tris (pH 9), 20 mM $(NH_4)_2SO_4$, 0.1% (wt/vol) Tween 20, 2 mM $MgCl_2$, 0.2 mM (each) dGTP, dATP, and dCTP, 0.6 mM dUTP, 50 pmol (each) of primers (5′-CCGAGTTTGATCCAAAAAGTTAC GAA-3′) and (5′-TAGCTCCTCATATGCCTTATTGAGTA-3′) (14, 15), 0.5 U of uracil-*N*-glycosylase (UNG; Boheringer Mannheim), and 1 U of *Taq* DNA polymerase (Eurogentec). Reaction mixtures were initially incubated for 10 min at 22°C to allow the UNG to destroy any amplified product containing deoxyuridine triphosphate that could have been carried over from the previous reactions. After denaturation at 94°C for 10 min, the samples were subjected to 50 cycles of 60 s at 94°C, 90 s at 56°C, and 90 s at 72°C, followed by a 5-min extension at 72°C (18). PCR products were then suspended in the sample buffer containing 1.2 M urea, 3.4% saccharose, 10 mM EDTA, and 0.002% bromophenol blue to inactivate the UNG, and 10 μl was analyzed on horizontal agarose gels in TAE buffer (40 mM Tris acetate, 2 mM Na_2 EDTA·$2H_2O$). Each amplification run contained a negative control (extraction buffer) and a positive control (DNA from 10 purified oocysts of *C. parvum*). For all fecal samples analyzed a 1/10 dilution of the lysate was amplified to dilute putative residual PCR inhibitors. Moreover, group 2 samples were amplified in duplicate by adding DNA from 10 oocysts of *C. parvum* to the reaction mixtures to ensure that negative results were not due to the copurification of PCR inhibitors.

Amplification of the 452-bp band specific to *C. parvum* was achieved for all naturally infected specimens from humans, calves, and goats (Fig. 1B), which demonstrates that the filtration procedure described is adapted to the fact that fecal material is a heterogeneous medium. Analysis of group 1 samples produced the 452-bp fragment for 10 of 10 specimens seeded with 1,000 oocysts per g and 8 of 10 specimens seeded with 100 oocysts per g (Fig. 1A). As a comparison, previous experiments aimed at determining the threshold of detection of *C. parvum* oocysts in formed human stools processed by a modified Ritchie concentration technique showed that a 100% detection rate was achieved with oocyst concentrations of 50,000 and 500,000 oocysts per g when using immunofluorescence and acid-fast detection methods, respectively (26). The procedure described in the present paper thus has a 50- to 500-fold increase in sensitivity compared with those of these commonly used methods. Enzyme immunoassays (EIAs) for the detection of *C. parvum* in stool specimens have recently been described. A first-generation EIA allowed for the detection of 3×10^5 oocysts per ml of calf feces (1). A commercially available assay was shown to be comparable to the immunofluorescence technique for the detection of oocysts in human stools (21). The only report comparing EIA and PCR with stool specimens demonstrated a 100-fold difference in sensitivity in favor of PCR (16). Therefore, the available data suggest that PCR is a more sensitive approach than EIA.

The strategy used by Laxer et al. (15) for the selection of the DNA sequence amplified in this study was designed to clone a *C. parvum*-specific gene, and indeed, PCRs performed with human DNA, as well as DNA from *Giardia lamblia*, *Toxoplasma gondii*, *Entamoeba histolytica*, *Plasmodium falciparum*, and *Trichomonas vaginalis* showed no cross-reactivity (15).

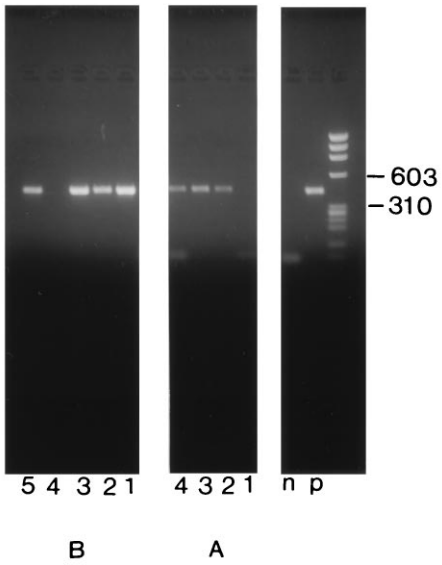

FIG. 1. Ethidium bromide-stained agarose gel of the products amplified by PCR from feces testing positive or negative for *C. parvum* by Ziehl-Neelsen staining. The apparent molecular sizes (in base pairs) of the size markers (*Hae*III-digested ϕX174 DNA) are given. p, positive control (DNA from 10 purified oocysts of *C. parvum* was used as a template for the PCR); n, negative control (the extraction buffer alone was used as template of the PCR). (A) Sample for DNA extraction that tested negative for *C. parvum*. Lane 1, analysis prior to oocyst addition; lane 2, same sample as that in lane 1, but seeded with *C. parvum* oocysts (100 oocysts per g); lane 3, same sample as that in lane 1, but seeded with *C. parvum* oocysts (1,000 oocysts per g); lane 4, same sample as that in lane 3, but the DNA was diluted 1/10 for PCR amplification. All PCR products in panel A were amplified in the same PCR run. (B) Analysis of stool specimens testing positive (lanes 1, 2, and 3) or negative (lane 4) for *C. parvum* by Ziehl-Neelsen staining. Lanes 1 and 4, human samples; lane 2, calf sample; lane 3, goat sample; lane 5, same sample as that in lane 4, but to which DNA extracted from 10 *C. parvum* oocysts was added to the reaction mixture prior to amplification. The PCR products in lanes 4 and 5 were amplified in the same PCR run.

Similarly, no amplification of DNA fragments was seen with DNA extracted from two bird isolates of *Cryptosporidium baileyi* (provided by M. Naciri and I. Varga) and from human isolates of *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Staphylococcus epidermidis*, *Enterococcus* spp., mixed anaerobic bacteria cultured from a human stool specimen, *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida tropicalis*, *Aspergillus fumigatus*, and *Fusarium* spp., all obtained at our institution (data not shown). These results, together with the absence of DNA amplification for group 2 specimens (16 stools; Fig. 1B) as well as group 1 specimens which were analyzed prior to the addition of oocysts (10 stools), indicate that the described method is specific for *C. parvum* DNA in stools.

Morgan et al. (17) amplified a 680-bp *C. parvum* DNA sequence from eight human stool samples containing 1×10^3 to 1.5×10^6 oocysts per g. However, only one specimen consisted of solid fecal material; therefore, one cannot anticipate the sensitivity of this procedure with nondiarrheic samples. Moreover, neither a stool disinfection procedure nor a UNG inactivation protocol was reported in that paper (17). Two other PCR methods that were also based on the DNA sequence amplified in the present study have been published recently. However, the first report by Leng et al. (16) consisted of the analysis of two bovine fecal samples only, and the second paper, by Balatbat et al. (2), described a PCR assay based on nested amplification, a technical approach that is not recommended for use in clinical laboratories (18) due to a major risk of contamination with PCR products. The method described herein thus contributes to the diagnosis of cryptosporidiosis in several ways: it includes a sodium hypochlorite disinfection step and a UNG inactivation protocol, it reproducibly identifies *C. parvum* in both formed and solid stools obtained from humans, and it is characterized in terms of its sensitivity. It is clear, however, that this approach to the diagnosis of cryptosporidiosis is complex, costly, and time-consuming compared to the techniques currently used in clinical laboratories. Therefore, it should be targeted toward selected patients and performed in laboratories having sufficient expertise in both parasitology and molecular biology. A prospective study of HIV-infected patients is now needed in order to study more precisely the natural history of cryptosporidiosis in AIDS patients and to determine the subpopulations of patients likely to benefit from this test strategy.

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