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Detection of *Cryptosporidium parvum* DNA in Human Feces by Nested PCR

A. BENIGNO BALATBAT, GEORGE W. JORDAN,* YAJARAYMA J. TANG, AND JOSEPH SILVA, JR.

Division of Infectious Diseases, University of California, Davis Medical Center, Sacramento, California 95817

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***Cryptosporidium parvum* is a coccidian protozoan that causes diarrhea in humans, often chronic and severe in patients with AIDS. Conventionally, diagnosis is made by concentration of stools followed by acid-fast staining (AF) or immunofluorescent staining. The threshold of detection in human stool specimens by these methods may require the presence of 50,000 (immunofluorescent staining) to 500,000 (AF) oocysts per g of stool. In this study, a nested PCR assay was developed to detect *C. parvum* DNA directly from stool specimens. After extraction of DNA from formalinized stool, a 400-bp fragment of *C. parvum* DNA was amplified with two 26-mer outer primers. The amplicon from this reaction was amplified with a second primer pair. With these nested primers, a 194-bp DNA fragment was amplified and confirmed as *C. parvum* DNA by internal probing with an enzyme-linked chemiluminescence system. This PCR-based test allowed the detection of 500 oocysts per g of stool or 100 ng of *C. parvum* DNA. Studies indicate that the primers utilized are specific for the DNA of *C. parvum*. DNA sequences were also detected in stool specimens from 4 of 28 patients previously reported negative by AF. In summary, a rapid, sensitive, and specific assay for the detection of *C. parvum* directly from stool specimens has been developed. This test has the potential for detecting asymptomatic infection, monitoring the response to therapy, and detecting the organism in environmental sources.**

Cryptosporidium parvum is a coccidian protozoan that causes diarrhea in humans, usually self-limited in immunocompetent hosts but severe and debilitating in immunocompromised hosts. Cryptosporidiosis is very common in patients with AIDS. In a study of human immunodeficiency virus-infected patients with diarrhea, 37.3% were found to be infected with *C. parvum* (1).

Commonly utilized coprodiagnostic methods involving stool concentration and visualization using acid-fast staining (AF) or immunofluorescent staining (IF) have a low sensitivity, especially in formed stools, in which the threshold for detection may require the presence of 50,000 (IF) to 500,000 (AF test) oocysts per g of stool (12). In a recent study of human immunodeficiency virus-infected patients with chronic diarrhea and negative stools by AF, *C. parvum* oocysts were detected in 11.6% of patients undergoing small bowel biopsy (5). The median infective dose of *C. parvum* has been found to be 132 oocysts in healthy volunteers and can be as low as 30 oocysts (2). Asymptomatic carriage can be significant and may cause person-to-person transmission. Therefore, development of sensitive diagnostic methods would have significant clinical implications.

A DNA sequence of *C. parvum* has been identified and successfully amplified by Laxer et al. using PCR (6). In this study, we report the detection of *C. parvum* DNA from oocysts directly in human stool specimens by a nested PCR technique. Our preliminary results indicate a significantly enhanced sensitivity over that of traditionally used methods.

MATERIALS AND METHODS

Extraction of DNA. DNA was extracted from purified *C. parvum* oocysts and from pure cultures of *Entamoeba histolytica*, a *Microsporidia* sp. (*Septata intestinalis*), *Blastocystis hominis* and *Giardia lamblia*, all obtained from the American Type Culture Collection, by a modification of the method of Kato et al. (4). One hundred microliters of the cell suspension (1×10^5 to 5×10^5 cells) was centrifuged at high speed ($7{,}000 \times g$) to pellet cells. The cells were then suspended in TES buffer (50 mM Tris [pH 8], 5 mM EDTA, 50 mM NaCl) containing 0.8% Sarkosyl and 100 μg of proteinase K per ml and incubated at 60°C for 2 h. After digesting with proteinase K, the supernatant was extracted twice with phenol-chloroform-isoamyl alcohol (24:24:1) and the DNA was precipitated with 2 volumes of ice-cold ethanol.

For the sensitivity studies, human stool specimens that tested negative for *C. parvum* by AF were spiked with *C. parvum* oocysts at concentrations of 100, 500, 1,000, and 10,000 oocysts per g of stool. The DNA from these spiked specimens was extracted as described above with the following modifications. Briefly, 100 mg of stool specimen was dissolved in 400 μl of TES buffer. The suspension was centrifuged at $2{,}000 \times g$ for 3 min to remove large particles. The supernatant containing the oocysts was then centrifuged at high speed ($7{,}000 \times g$) to pellet oocysts. The pelleted oocysts were resuspended in 5.25% sodium hypochloride and washed twice in distilled water. The washed pellet was suspended in 240 μl of TES buffer containing 0.8% Sarkosyl and 100 μg of proteinase K per ml and treated as described above. The precipitated DNA was quantitated as described elsewhere (3).

DNA amplification from stool specimens and from pure cultures. A nested PCR approach was used to amplify a 194-bp DNA fragment of *C. parvum* as shown in Fig. 1. This segment of *C. parvum* DNA as well as the outer primers and probe correspond to sequences described by Laxer et al. (6). The PCR method of Mullis and Faloona (8) was used for amplification with the thermostable DNA polymerase (rTaq; Perkin-Elmer Cetus). For the first amplification reaction the outer primers, BB-1 and BB-2, were used. The reaction mixtures were prepared in PCR buffer (50 mM KCl, 20 mM Tris-HCl, 2.5 mM $MgCl_2$, 100 μg of bovine serum albumin per ml, pH 8.4) and contained, per reaction, 20 pmol of the respective primers, a 0.1 mM concentration of each of the 2′-deoxynucleoside 5′-triphosphates, 2 U of recombinant thermostable DNA polymerase, and 5 μl of the purified DNA. For the second round of amplifications, the reaction mixture was prepared as described above, except that the inner primers, BB-3 and BB-4, were used and 5 μl of the amplified product from the first PCR was used as the source of DNA. The reaction mixtures were covered with 150 μl of mineral oil (Sigma Chemical, St. Louis, Mo.) to prevent evaporation, and the reactions were initiated by denaturing the DNA at 95°C for 1 min, followed by annealing of the primers at 45°C for 2 min, with extension at 72°C for 3 min. Amplification was done for 35 cycles in a thermal cycler (MJ Research). Negative controls consisted of a blank containing all PCR reagents but no DNA.

Detection of amplified products. Amplification products were visualized by running 12 μl of the reaction mixture in 2% agarose gels in Tris-borate-EDTA buffer. Size markers included in all gels were the 123-bp DNA ladder (Bethesda Research Laboratories, Grand Island, N.Y.). Gels were run at a constant 110 V for 90 min. Gels were stained in an ethidium bromide solution (0.5 μg/ml) for 30 min, destained for 30 min, and photographed under UV light with a Land camera (Polaroid, Cambridge, Mass.).

* Corresponding author. Mailing address: Division of Infectious and Immunologic Diseases, University of California, Davis Medical Center, 4301 X St., Professional Building, Suite 2410, Sacramento, CA 95817. Phone: (916) 734-3741. Fax: (916) 734-7766.

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                                  —>       BB-1
421 TGAAGGTGGT GAGAATGGAA CTGCCGAGTT TGATCCAAAA AGTTACGAAG AAAAATCCAG
    ACTTCCAGGA CTCTTACCTT GACGGCTCAA ACTAGGTTTT TCAATGCTTC TTTTTAGGTC

                                  —>      BB-3
481 AGATCAAAAG ACAAAGAATA CTCAAAGCGA AGATGACCTT TTGATTTGTT TATGAAGGAG
    TCTAGTTTTC TGTTTCTTAT GAGTTTCGCT TCTACTGGAA AACTAAACAA ATACTTCCTC

541 GTTAATGAAG CAAACAGTAA AAGCACTAAC CAAGGAAAAA ACATTGGCAG AAATAATCAT
    CAATTACTTC GTTTGTCATT TTCGTGATTG GTTCCTTTTT TGTAACCGTC TTTATTAGTA

601 TCAAAAAGGG TCAAAATATA CAAAGTTATT AATTGAAGGA AAAATAAAGG AAGCCATTAG
    AGTTTTTCCC AGTTTTATAT GTTTCAATAA TTAACTTCCT TTTTATTTCC TTCGGTAATC

                                —>    BB-5
661 TAGCTCATAT GGCCAATGAT GAATTAACCT ATAGGAACCT CAGAAGAAGA AATCCTACGT
    ATCGAGTATA CCGGTTACTA CTTAATTGGA TATCCTTGGA GTCTTCTTCT TTAGGATGCT
                                                  BB-4      <—

721 CTAACTTCAC GTGTGTTTGC CAATGCATAT GAAGTTATAG GGATACCAGT CGATTCTGAT
    GATTGAAGTG CACACAAACG GTTACGTATA CTTCAATATC CCTATGGTCT GCTAAGACTA

781 GATTCTGTGA TTGGTAAAAA GTATAGAAGC TGTCATTATT GTACCACCCT GATAAGACAA
    CTAAGACACT AACCATTTTT CATATCTTCG ACAGTAATAA CATGGTGGGT CTATTCTGTT

841 GTCATGAAAG GCTAGAGAAG CGTTTGAAAT ACTCAATAAG GCATATGAGG AGCTACAAAA
    CAGTACTTTC CGATCTCTTC GCAAACTTTA TGAGTTATTC CGTATACTCC TCGATGTTTT
                                                  BB-2        <—

BB-1/BB-2:  Outer primers- amplicon size:  402 bp

BB-3/BB-4:  Inner primers- amplicon size:  194 bp

BB-5: Probe
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FIG. 1. *C. parvum* DNA sequence targeted for amplification.

Southern blot analysis. PCR products were confirmed as *C. parvum* by internal probe hybridization. A nonradioactive method employing horseradish peroxidase, a biotin-labeled oligomer, and an enzyme chemiluminescence detection system (Amersham, Arlington Heights, Ill.) was used as previously described (3).

RESULTS

Amplification of *C. parvum* DNA. We developed a nested PCR assay to detect *Cryptosporidium* oocysts directly in stool specimens. With a pair of outer primers (BB-1 and BB-2) the expected 400-bp amplicon was obtained from DNA extracted from purified oocysts and from oocysts present in stool specimens (Fig. 2). Amplification of this amplicon with the inner primers (BB-3 and BB-4) yielded the expected 194-bp DNA fragment. The specificity of the product was confirmed by Southern blot analysis with a biotin-labeled internal probe (BB-5). As can be seen in Fig. 4 (lanes 9 and 17), DNA from purified oocysts hybridized with the probe.

Sensitivity of PCR in detecting *C. parvum* DNA. The sensitivity of this system in detecting *C. parvum* was determined with two experiments. In the first set of experiments, *Cryptosporidium* oocysts at concentrations ranging from 100 to 10^4 were inoculated into 1 g of stool obtained from a healthy volunteer. This nested PCR system allowed the detection of *C. parvum* when as few as 500 oocysts per g of stool were present (Table 1 and Fig. 2). This experiment was repeated three times and consistently detected 500 oocysts per g of stool; no amplification product was detected with 100 oocysts per g. DNA extracted from the unspiked stool could not be amplified by the nested PCR method for *C. parvum*; however, the 16S rRNA gene of enteric bacteria was amplifiable, indicating the lack of inhibitors of *Taq* polymerase in the extract (data not shown). We also determined the sensitivity of the PCR assay in amplification reactions with *C. parvum* DNA at concentrations ranging from 10 ng to 1 μg. In these experiments, as little as 100 ng of cryptosporidial DNA was detected (Table 1).

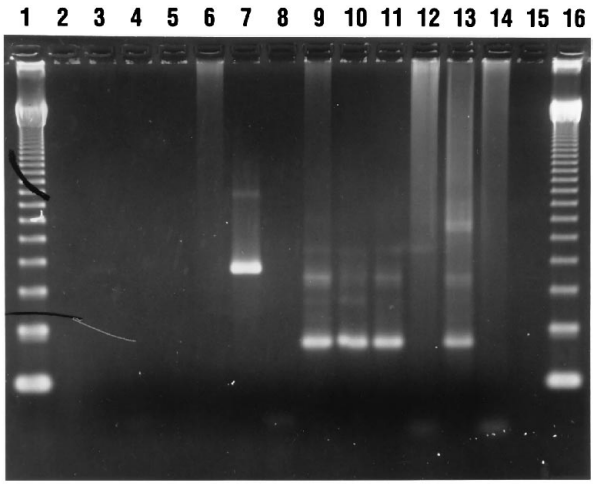

FIG. 2. Sensitivity of PCR in detecting *C. parvum* DNA. Lanes 1 and 16, 123-bp DNA marker ladder; lanes 3 to 6, DNA from stool spiked with 10^4, 10^3, 500, and 10^2 *C. parvum* oocysts, respectively, and amplified with the outer primers; lanes 9 to 12, PCR products after amplification of the first amplicon with the inner primers (10^4, 10^3, 500, and 10^2 oocysts, respectively); lanes 7 and 13, DNA from *C. parvum* oocysts after outer and both outer and inner primer amplification, respectively; lanes 8 and 14, negative control (containing all PCR reagents but no target DNA added).

Specificity of the PCR. To further establish the specificity of our PCR system, amplification reactions were carried out with DNA from other common intestinal parasites. As can be seen in Fig. 3A, the nested PCR was able to differentiate *C. parvum* from *E. histolytica*, *G. lamblia*, *B. hominis*, and *S. intestinalis*. No amplification products of the expected size were obtained with the DNA extracted from any of these protozoans, and

TABLE 1. Sensitivity of PCR in detecting *C. parvum*

Prepn tested	Detection by:		
	Amplification		Conventional tests[a]
	Outer primer	Nested	
Oocysts[b] (no. [10^2])			
1	−	−	ND
5	−	+	ND
10	−	+	−
100	−	+	+
5,000	ND	ND	+
50,000	ND	ND	+
DNA[c] (ng)			
10	−	−	
100	−	+	NA
500	−	+	NA
1,000	+	+	NA

[a] These results are taken from the literature and cited in reference 2. ND, not done; NA, not applicable.
[b] *C. parvum* oocysts were inoculated into stools from asymptomatic patients. The DNA was extracted and amplified as indicated in the text.
[c] DNA from *C. parvum* was amplified as described in the text.

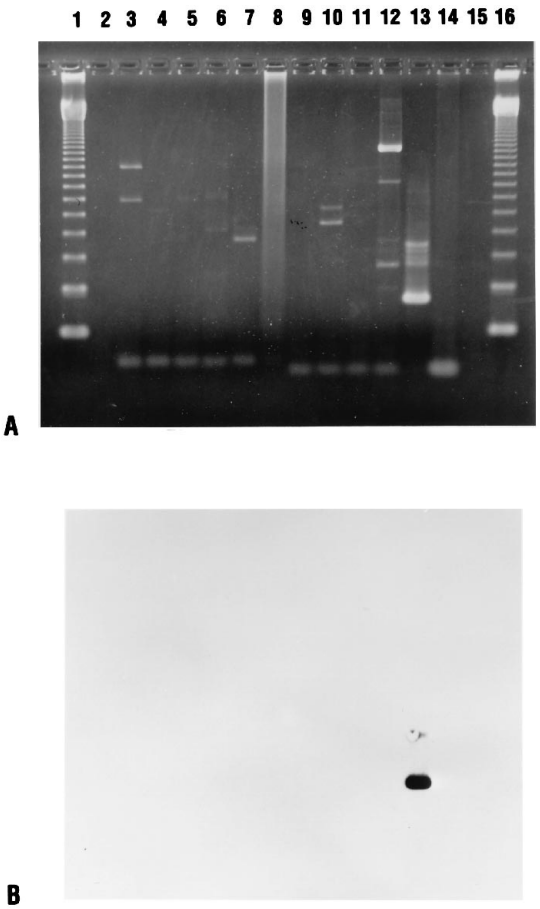

FIG. 3. Specificity of the PCR assay in differentiating *C. parvum* from other intestinal parasites. (A) Ethidium bromide-stained 2% agarose gel of PCR products; (B) Southern blot analysis of products in panel A. Lanes 1 and 16, 123-bp DNA ladder; lanes 3 and 9, DNA from *E. histolytica*; lanes 4 and 10, DNA from *G. lamblia*; lanes 5 and 11, DNA from *B. hominis*; lanes 6 and 12, DNA from *S. intestinalis*; lanes 7 and 13, DNA from *C. parvum*; lanes 8 and 14, negative controls (containing all PCR reagents but no target DNA).

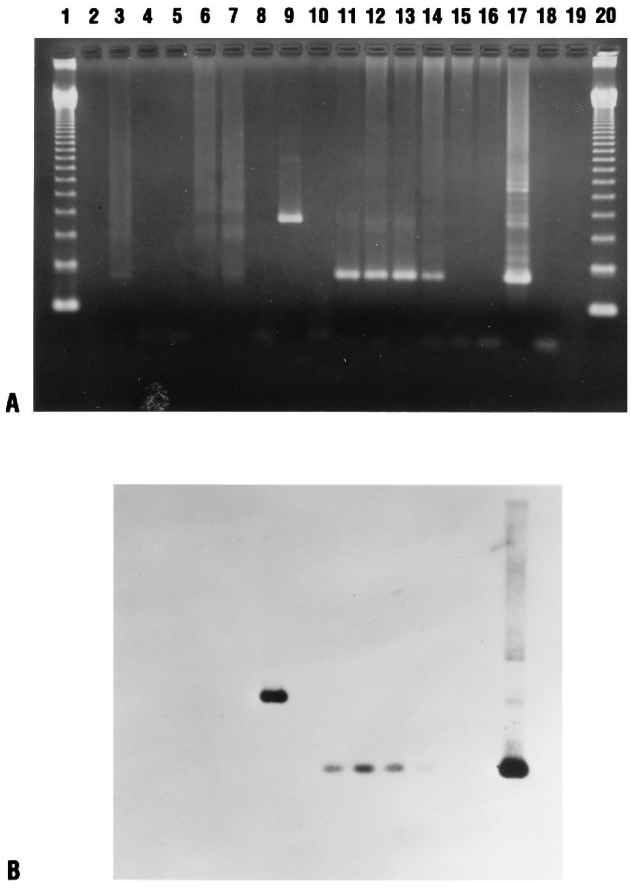

FIG. 4. Ethidium bromide-stained 2% agarose gel with PCR products of DNA from clinical specimens (A) in Southern blot analysis (B). Lanes 1 and 20, 123-bp DNA marker ladder; lanes 3 to 8, DNA from symptomatic patients amplified with outer primers BB-1 and BB-2; lanes 11 to 16, PCR products after amplification of the first amplicon with inner primers BB-3 and BB-4 (expected size, 194 bp); lanes 9 and 17, DNA from 10^3 *C. parvum* oocysts after outer and both outer and inner primer amplification, respectively; lanes 10 and 18, negative controls (containing all reagents but no target DNA added).

these samples remained negative when probed with the internal probe (Fig. 3B).

Amplification of clinical specimens. To determine the feasibility of our PCR technique as a clinical assay, we extracted and amplified DNA from 28 stool specimens from patients with AIDS who had diarrhea but whose stools had tested negative by the AF test. *C. parvum* DNA sequences were detected in four of these patients after amplification with the inner primers (Fig. 4A). These products were confirmed as *C. parvum* by Southern blot analysis (Fig. 4B).

DISCUSSION

Cryptosporidiosis is recognized as one of several emerging infectious diseases. First identified as causing human disease in 1976 (9), *C. parvum* causes sporadic gastrointestinal disease in healthy hosts but a very severe cholera-like illness in immunocompromised hosts. In 1993, *C. parvum* caused the largest waterborne outbreak in the United States (Milwaukee, Wis.), affecting as many as 400,000 people (7), with biliary cryptosporidiosis resulting in human immunodeficiency virus-infected people, who are particularly at risk (10). Although current laboratory methods are generally considered adequate to detect high concentrations of oocysts, they fail to detect cases of cryptosporidiosis in many immunocompromised patients.

In this report we describe a rapid, sensitive, and specific method for the direct detection of *C. parvum* in stool specimens by PCR. PCR-based assays have previously been used by others to identify *C. parvum*. In those studies, amplification reactions were performed on DNA extracted from purified oocysts or paraffin-embedded tissues (6, 13). We have applied this technique to directly detect *C. parvum* in stool specimens. The specific PCR amplification from fecal extract eliminates the need for laborious concentration procedures with the subsequent loss of oocysts which occurs during purification (12).

The sensitivity of current diagnostic methods (AF and IF) for detecting *C. parvum* oocysts in human stools has been reported to be 10,000 oocysts per g of watery stool, while in formed stools 50,000 or 500,000 oocysts are required for a positive IF or AF test, respectively (12). Therefore, more-sensitive methods are clearly needed to identify asymptomatic or chronic carriers in whom the burden of oocysts is below the threshold for detection.

Webster et al. (13) have reported the detection of 2,000 oocysts by PCR after amplification of DNA extracted from purified oocysts. We have increased this sensitivity further by

employing a nested primer technique applied directly to stool extracts to detect as few as 500 oocysts per g of stool, which represents a 100-fold increase in sensitivity compared with that of the IF method. The degree of sensitivity achieved in this study may be particularly useful in identifying and diagnosing those symptomatic patients with stools negative by the traditional diagnostic methods. Recently, a sensitivity of 100 purified sporozoites has been achieved by a similar technique (11).

We determined the specificity of our system to the DNA extracted from other common intestinal parasites (*G. lamblia*, *B. hominis*, *E. histolytica*, and *S. intestinalis*). Nonspecific amplification products of various sizes were observed with the DNA from some of these organisms; however, the oligonucleotide probe, BB-5, hybridized only to the PCR product derived from *C. parvum*. Using the outer primers, Laxer et al. (6) were unable to amplify DNA from *Toxoplasma gondii*, *Trichomonas vaginalis*, or *Plasmodium falciparum*. Although the function of the target DNA segment is unknown (6), these results suggest that it is not shared by these other species of protozoans.

The sensitivity of the PCR technique was also tested at the clinical level. We have demonstrated *C. parvum* oocysts in the stools of four AIDS patients with diarrhea whose stools were negative by the AF test. This assay may contribute to the identification of a population of patients who are carriers of *C. parvum* (in either the presence or absence of active clinical disease) at a threshold below that detectable by the current diagnostic tests. These findings may have significant clinical implications and may contribute to a better understanding of the epidemiology of cryptosporidiosis. Another potential application of this PCR assay could be in environmental studies and testing of water treatment plants as well as in monitoring responses to new therapeutic drugs.

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