UNIVERSITY OF AMSTERDAM

UvA-DARE (Digital Academic Repository)

Specific PCR assay for direct detection of intestinal microsporidia Entyrocytozoon bieneusi and Encephalitozoon intestinalis in fecal specimens from human immunodeficiency virus-infected patients

Ombrouck, C.; Ciceron, L.; Biligui, S.; Brown, S.; Marechal, P.; van Gool, T.; Datry, A.; Danis, M.; Desportes-Livage, I.

Publication date
1997

Published in
Journal of Clinical Microbiology

Link to publication

Citation for published version (APA):
Ombrouck, C., Ciceron, L., Biligui, S., Brown, S., Marechal, P., van Gool, T., Datry, A., Danis, M., & Desportes-Livage, I. (1997). Specific PCR assay for direct detection of intestinal microsporidia Entyrocytozoon bieneusi and Encephalitozoon intestinalis in fecal specimens from human immunodeficiency virus-infected patients. *Journal of Clinical Microbiology*, *35*, 652-655.

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
UvA-DARE is a service provided by the library of the University of Amsterdam (https://dare.uva.nl)

Download date:11 Mar 2023

Journal of Clinical Microbiology, Mar. 1997, p. 652–655 Vol. 35, No. 3
0095-1137/97/$04.00+0
Copyright © 1997, American Society for Microbiology

Specific PCR Assay for Direct Detection of Intestinal Microsporidia *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* in Fecal Specimens from Human Immunodeficiency Virus-Infected Patients

CECILE OMBROUCK,[1]* LILIANE CICERON,[1] SYLVESTRE BILIGUI,[1] SPENCER BROWN,[2] PIERRE MARECHAL,[3] TOM VAN GOOL,[4] ANNICK DATRY,[1] MARTIN DANIS,[1] AND ISABELLE DESPORTES-LIVAGE[1]

Centre Hospitalo-Universitaire de la Pitié-Salpêtrière, Unité INSERM 313, 75013 Paris,[1] Laboratoire de Cytométrie, Institut des Sciences Végétales, CNRS, 91198 Gif-sur-Yvette,[2] and Laboratoire de Biologie Parasitaire, Museum National d'Histoire Naturelle, URA CNRS 114, 75231 Paris Cedex 05,[3] France, and Department of Medical Microbiology, Academic Medical Center, University of Amsterdam, 1105 AZ, Amsterdam, The Netherlands[4]

Received 19 July 1996/Returned for modification 21 October 1996/Accepted 16 December 1996

A routine assay based on the PCR was developed for the detection of *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* in fecal samples. Two oligonucleotide primer pairs from a conserved region in the small-subunit rRNA genes of *E. bieneusi* (primer pair V1 and EB450) and *E. intestinalis* (primer pair V1 and SI500) were used to amplify microsporidian DNA. We achieved specific amplification of a 382-bp DNA fragment in *E. intestinalis* and a 353-bp DNA fragment in *E. bieneusi*. Boiling of the samples appeared to be most effective for DNA extraction. Fecal samples containing fewer than 10 microsporidia gave a positive result in the PCR assay. Fecal specimens from 30 human immunodeficiency virus-infected patients with microsporidiosis and fecal specimens from 42 patients suspected of having microsporidiosis were investigated by the PCR assay. The PCR assay was validated against standard staining methods (the Uvitex 2B and Chromotrope 2R staining methods) and immunofluorescence assay specific for *E. intestinalis*. This comparative study has shown that PCR improved species determination and can thus be considered a fast and reliable method for the detection and identification of each intestinal species.

Although they are usually reported in animals, microsporidia have recently been recognized to be the cause of infections in humans, especially in immunocompromised patients. In a recent study, intestinal microsporidia were found in up to 39% of patients with AIDS and diarrhea and represented the most common organisms detected among enteric pathogens (12). The two species *Enterocytozoon bieneusi* (5), the most commonly identified species (2, 15), and *Encephalitozoon intestinalis* (10) are responsible for intestinal pathology in human immunodeficiency virus-infected patients with clinical signs of disease including chronic diarrhea, nausea, malabsorption, and severe weight loss; *E. intestinalis* is also responsible for disseminated infection (2, 15).

Diagnosis of intestinal microsporidiosis is hampered by the small size of the spores released in feces. Spores of *E. bieneusi* usually measure 1.5 by 1 μm, whereas the average size of spores of *E. intestinalis* is 2.2 by 1.2 μm (15). Attention has been focused toward the development of reliable, sensitive, and efficient methods for detecting intestinal microsporidia in clinical specimens. For that purpose, two staining methods have been proposed: staining with a modified trichrome stain, Chromotrope 2R (11, 21), and fluorescent brightener (Uvitex 2B; calcofluor) staining of the chitin present in the microsporidian spore wall (18, 19). Indirect immunofluorescent-antibody staining methods with polyclonal and monoclonal antibodies have also been used to detect microsporidia (1, 25). Presently, routine detection of microsporidia in stool specimens is usually performed by the trichrome stain or Uvitex 2B method or even a combination of these methods (6). However, transmission electron microscopy is still the sole method which allows species identification (15). A reliable, rapid technique allowing for the differentiation between *E. bieneusi* and *E. intestinalis* in fecal samples would be useful for the clinical management of patients with severe symptoms. Indeed, *E. intestinalis* responds to albendazole therapy, whereas for *E. bieneusi*, no convincing therapy is available (2, 18). The PCR has already been applied to the detection of microsporidia in intestinal biopsy, stool, and bile specimens and allowed for the detection of microsporidia (4, 7, 8, 22, 24). However, a PCR assay for the routine detection of microsporidia in clinical specimens is not yet available.

The purpose of this study was to develop a PCR assay for the rapid analysis of fecal samples from patients with intestinal microsporidiosis. The specificity and sensitivity of this assay were evaluated by comparison with conventional staining methods (the Chromotrope 2R and Uvitex 2B staining methods) and immunofluorescence assay (IFA).

MATERIALS AND METHODS

Microsporidian specimens. Spores of the two microsporidian species were used. Spores of *E. intestinalis* were obtained from cultures in rabbit kidney cells (RK13), as described by van Gool et al. (17). Parasite spores were harvested weekly and were washed and resuspended in phosphate-buffered saline (PBS). Spores of *E. bieneusi* were isolated from feces by flow cytometry as described before (3).

Stool specimens and DNA extraction. The fecal samples were clinical specimens submitted for routine parasitological analysis. Thirty samples from human immunodeficiency virus-infected patients in whose feces microsporidian spores were detected were initially studied by PCR. Microsporidia were detected by stool examinations with Uvitex 2B stain (18) and modified trichrome stain (11). Additionally, 42 stool samples examined for the presence of microsporidia by the Uvitex 2B and Chromotrope 2R staining methods were coded and tested blind

* Corresponding author. Mailing address: Unité INSERM 313, CHU Pitié-Salpêtrière, 91, Bld. de l'Hôpital, 75013 Paris, France. Phone: 33 1-45 83 46 84. Fax: 33 1-45 83 88 58.

by PCR. All samples were also screened by a double staining method by combining an IFA with a polyclonal antibody specific for *E. intestinalis* and Uvitex 2B stain as described before (13).

Fecal samples were diluted in PBS with 10% Formol (vol/vol) and were filtered through a 50-μm-pore-size filter. After centrifugation at 2,500 × *g*/min for 5 min, the pellet was resuspended in PBS. Ten microliters of the final dilution was applied to glass slides for staining by standard techniques and IFA. One hundred microliters of the same solution was washed three times in PBS. The washed pellet was resuspended in 1 ml of PBS, and pellets which were rich in fecal material were diluted 10-fold.

Ten microliters of the final stool solution was used for DNA extraction. DNA was released by heating microsporidia suspended in PBS for 10 min at 100°C by the method of van Eys et al. (16). For each PCR assay with a fecal solution, a simultaneous amplification was performed with a sample of each fecal solution spiked with *E. intestinalis* or *E. bieneusi* spores in order to control for false-negative reactions due to the presence of inhibitory substances. Purified spores diluted in PBS were used as positive controls. All samples were subjected to DNA extraction before being tested by PCR (10 μl per reaction vial).

DNA primers and PCR amplification. The primers V1 (5′-CACCAGGTTG ATTCTGCCTGAC-3′) and EB450 (5′-ACTCAGGTGTTATACTCACGTC -3′) described by Zhu et al. (24) were used to amplify *E. bieneusi* DNA. The primers V1 and SI500 (5′-CTCGCTCCTTTACACTCGAA-3′) described by Weiss et al. (22) were used to amplify *E. intestinalis* DNA.

Amplification of DNA was performed in a total volume of 50 μl. The PCR mixture consisted of 10× reaction buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin), 200 μM (each) deoxynucleotide triphosphate, 300 pM (each) primer, and 0.5 U of *Taq* polymerase (ATGC, Noisy le Grand, France). The mixture was then covered with 80 μl of mineral oil. For the two sets of primers, one amplification cycle consisted of an initial denaturation of target DNA at 94°C for 10 min, followed by denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min, and elongation at 72°C for 3 min. The last elongation step was extended to 10 min. Samples were amplified through 35 consecutive cycles. PCR products were detected by agarose gel electrophoresis and ethidium bromide staining. Negative control reactions with the reagents were performed with each batch of amplifications and consisted of tubes containing distilled water in place of the DNA samples.

Southern hybridization with a radioactive system. Amplified DNA was transferred from the agarose gel to a positively charged nylon membrane (Hybond-N^+; Amersham, Les Ulis, France). The internal 30-mer oligonucleotide primer EB150 (5′-TGTTGCGGTAATTTGGTCTCTGTGTGTAAA-3′) described by Zhu et al. (24), which was used to confirm that the amplified DNA was from *E. bieneusi* (24), was end labelled with [γ-^{32}P]ATP (ICN, Orsay, France) and T4 polynucleotide kinase. Prehybridization was carried out in hybridization buffer for at least 1 h at 50°C, followed by an overnight hybridization at 50°C in the same buffer containing the labelled probe at a concentration of 0.5×10^6 cpm/ml. Following hybridization, the blot was then washed twice for 15 min each time in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% sodium dodecyl sulfate (SDS) at room temperature and once for 3 min at 55°C in the same buffer before being sealed in Saran wrap and exposed to Hyperfilm ECL (Amersham) for 2 to 16 h at −80°C.

Southern hybridization with a nonradioactive system. Internal oligonucleotide probes were labelled with digoxigenin-11-dUTP by using the DIG Oligonucleotide Tailing Kit (Boehringer Mannheim, Meylan, France). EB150 was used to confirm DNA amplification from *E. bieneusi* and the oligonucleotide sequence for the *E. intestinalis* probe was 5′-TGGTAGTTAGGGTAATGGCCTAACTA GGCG-3′. Prehybridization was for at least 2 h at 50°C with hybridization buffer containing 5× SSC, 0.1% (wt/vol) *N*-lauroylsarcosine, 0.02% (wt/vol) SDS, and 1% blocking reagent, as recommended by Boehringer Mannheim. Hybridization was carried out overnight at 50°C in the same buffer with 0.5 pmol of the labelled probe per ml. The blot was then washed twice for 5 min each time in 2× SSC–0.1% SDS at room temperature and twice for 15 min in 0.5× SSC–0.1% SDS at 50°C. Bound digoxigenin-11-dUTP-labelled oligonucleotides were detected by using the DIG Luminescent Detection Kit (Boehringer Mannheim). The membrane was autoradiographed on Hyperfilm ECL (Amersham) for 1 to 15 min.

DNA subcloning and sequencing. The amplification products were purified by using the Prepa gene kit (Promega, Charbonnieres, France). They were then cloned by using the pGEM-T Vector System II kit (Promega) and were sequenced by a commercial company (ESGS, Montigny le Bretonneux, France). For each microsporidium, four independent small-subunit (SSU) rRNA gene clones were sequenced to eliminate PCR misincorporation artifacts. The homologies of the SSU rRNA gene sequences that were obtained to the other microsporidial SSU rRNA sequences present in GenBank were compared by using Genetics Computer Group software.

RESULTS

The positions of primer pairs V1-EB450 and V1-SI500 within the sequence of the SSU rRNA genes predicted that a 353-bp fragment and a 382-bp fragment would be generated following PCR of *E. bieneusi* and *E. intestinalis*, respectively.

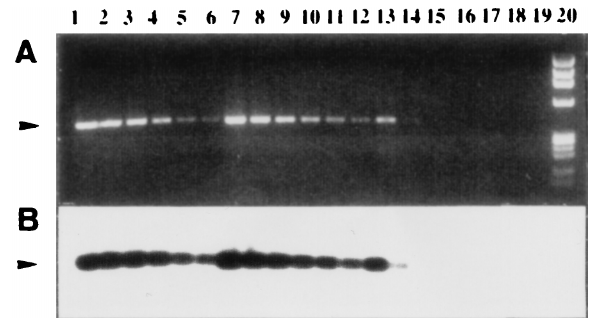

FIG. 1. Determination of the sensitivity of the PCR with suspensions of *E. intestinalis* and detection of *E. intestinalis* in fecal specimens. (A) Analysis by agarose gel electrophoresis. (B) Southern blot analysis and hybridization with a digoxigenin-11-dUTP-labelled, 30-mer probe. Lanes 1 to 6, 10^4, 10^3, 10^2, 10, 5, and 1 spore of *E. intestinalis*, respectively (made from a series of 10- and 2-fold dilution steps of an *E. intestinalis* suspension); lanes 7 to 12, negative stool specimen spiked with *E. intestinalis* at final concentrations of 10^4, 10^3, 10^2, 10, 5, and 1 spore per PCR vial, respectively; lanes 13 and 14, two stool samples found to be positive for *E. intestinalis* in our study; lane 15, spores of *E. bieneusi* isolated by flow cytometry; lanes 16 and 17, stools containing *E. bieneusi*; lanes 18 and 19, stools negative for microsporidia; lane 20, *Hae*III-digested ϕX174 DNA as a molecular size marker. Arrowheads indicate the 382-bp product obtained with primers V1-SI500 and by hybridization to the internal oligonucleotide probe.

Fragments of the appropriate size were visualized following PCR of released DNA obtained from parasites or stool specimens (Fig. 1 and 2).

Positive signals obtained by Southern hybridization of PCR products from *E. intestinalis* with the internal probe confirmed that the 382-bp amplified sequence was part of the SSU rRNA gene of *E. intestinalis* (Fig. 1B, lanes 1 to 14). Positive signals obtained by Southern hybridization of PCR products from *E. bieneusi* with the internal probe EB150 confirmed that the 353-bp amplified sequence was part of the SSU rRNA gene of *E. bieneusi* (Fig. 2B and C). Moreover, sequences of the SSU rRNA genes of both microsporidia corresponded to previous published sequences; GenBank database accession numbers are L16868 (*E. bieneusi*) (9) and U09929 (*E. intestinalis*) (20). No cross-reactivity was detected between the *E. intestinalis* primers and spores of *E. bieneusi* (Fig. 1, lanes 15 to 17) or between the *E. bieneusi* primers and spores of *E. intestinalis* (Fig. 2, lane 15). No signals were observed for fecal specimens negative for microsporidia but containing other pathogens (*Giardia intestinalis*, *Crypstosporidium* sp., *Blastocystis hominis*, and yeasts).

The sensitivity of the PCR assay was determined by adding mixtures containing decreasing amounts of spores to the reaction vials. Our results indicate that one spore of *E. intestinalis* could be amplified by using the V1-SI500 primers and gave a weak band on agarose gels (Fig. 1A, lane 6) which was clearly positive after hybridization with an internal digoxigenin-labelled probe (Fig. 1B, lane 6). In order to determine the sensitivity of the assay for the detection of *E. intestinalis* in fecal samples, a fecal specimen from a healthy human was divided into 10-μl samples and was seeded with serial dilutions of *E. intestinalis* spores. One spore per 10 μl per PCR assay could be detected on ethidium bromide-stained gels (Fig. 1A, lane 12) and could be confirmed by hybridization analysis (Fig. 1B, lane 12). The results for the two fecal samples found to be positive for *E. intestinalis* in our study are presented in Fig. 1, lanes 13 and 14.

The results of sensitivity experiments indicated that PCR products generated with 1,000 spores could be visualized on the agarose gel (Fig. 2A, lane 3). After hybridization analysis

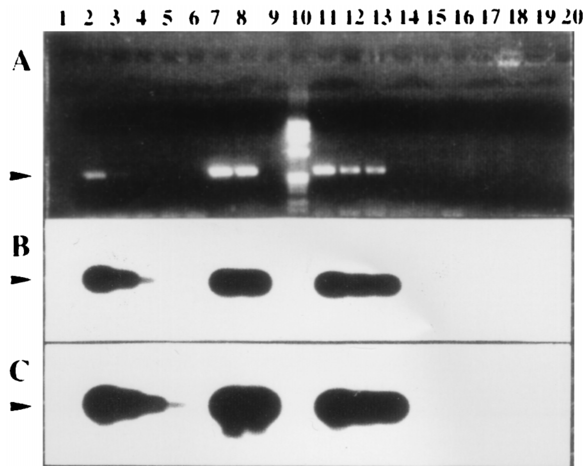

FIG. 2. Determination of the sensitivity of the PCR with suspensions of *E. bieneusi* and detection of *E. bieneusi* in fecal specimens. (A) Analysis of the PCR products by agarose gel electrophoresis. (B) Southern blot and hybridization with [γ-^{32}P]ATP-labelled EB150 probe; the time of exposure of the film after hybridization was 2 h. (C) Southern blot and hybridization with [γ-^{32}P]ATP-labelled EB150 probe; the time of exposure of the film after hybridization was 16 h. Lanes 2 to 6, 10^4, 10^3, 10^2, 10, and 1 spore of *E. bieneusi*, respectively (made from a series of 10-fold dilution steps of an *E. bieneusi* suspension isolated by flow cytometry); lanes 7 and 8, stools containing *E. bieneusi*; lane 9, negative stool; lane 10, *Hae*III-digested ϕX174 DNA as a molecular size marker; lanes 11 and 12, a stool positive for *E. bieneusi* nonspiked (lane 12) and spiked with 10^4 spores of *E. bieneusi* (lane 11); lane 13 and 14, a negative stool nonspiked (lane 14) and spiked with 10^4 spores (lane 13); lane 15, spores of *E. intestinalis*; lanes 16 to 20, stools negative for microsporidia. Arrowheads indicate the 353-bp product obtained with primers V1-EB450 and by hybridization to the EB150 oligonucleotide probe (lanes 2 to 8 and 11 to 13).

with the ^{32}P-labelled EB150 probe, the assay could detect between 10 and 100 spores per 10 μl per PCR assay, according to the time of exposure of the film after hybridization (Fig. 2B, lane 4; Fig. 2C, lane 5). One hundred spores could be detected after 2 h of exposure (Fig. 2B, lane 4), whereas 10 spores were detected after 16 h of exposure (Fig. 2C, lane 5). One hundred spores could be detected when the digoxigenin-labelled EB150 probe was used (data not shown).

A portion of each fecal sample was spiked with spores to detect any false-negative results caused by components which interfered with the PCR. When spiking showed that interference was present (when spiked samples were negative), we proceeded to test a 10-fold dilution. For all the stool specimens tested, this procedure was sufficient to restore PCR amplification. Representative results for *E. bieneusi* are presented in Fig. 2A, lanes 11 to 14. One fecal sample was a true negative for *E. bieneusi*, since the nonspiked sample was negative (lane 14) and the spiked sample was positive (lane 13). The second fecal sample was truly positive for *E. bieneusi*, since the nonspiked sample was positive (lane 12) and was confirmed to originate from this species by Southern analysis with the internal probe EB150 (Fig. 2B and C, lanes 12).

A total of 72 fecal specimens were analyzed by PCR, standard staining methods, and IFA. Thirty-four specimens were found to be positive by PCR and standard staining methods; however, differences in the species detected were observed between the methods. According to conventional staining methods, 28 fecal samples were supposed to contain *E. bieneusi* spores alone, whereas 5 stool specimens were supposed to contain the two species on the basis of morphologic differences. Of the five stool specimens supposed to contain the two species of microsporidia, only one was confirmed to be positive for the two species by PCR. Therefore, 32 fecal specimens were shown to contain only *E. bieneusi* by PCR, whereas 28 specimens were shown to contain only *E. bieneusi* by staining methods. This result was also confirmed by our IFA technique specific for *E. intestinalis*. However, there was a correlation between all the methods for the fecal sample containing only *E. intestinalis.*

DISCUSSION

Rapid and practical means for the specific detection of intestinal microsporidian infections are still lacking. A rapid, sensitive, and specific test for *E. bieneusi* and *E. intestinalis* would be of great value because of the clinical importance of those pathogens and their differential responses to drug therapy.

Detection of *E. bieneusi* and *E. intestinalis* in stool samples has already been performed. Restriction digests of the PCR products enabled those species to be differentiated on the basis of the sizes of their amplicons. However, the DNA extraction method used by those investigators appeared to be time-consuming (7).

The aims of this study were to develop a simple method for extracting the microsporidian DNA present in fecal specimens and to develop a specific and sensitive PCR with primers based on the sequences of microsporidian SSU rRNA genes. Preliminary data suggested the potential use of the PCR with primer sets V1-EB450 and V1-SI500, which were used as tools for the specific and sensitive detection of *E. bieneusi* and *E. intestinalis* in fecal specimens (14).

Concerning DNA extraction, boiling of the samples has been shown to be the simpler and more economical method for releasing DNA from bacteria (16). It also appears to be the most attractive and efficient method for the extraction of microsporidian DNA due to its rapidity and simplicity. This procedure provided sufficient amounts of pure DNA to yield amplified products from microsporidia in all the stool specimens tested in this study.

Our PCR assay enabled the direct detection of each species from fecal samples. Inhibitory compounds in feces may interfere with amplification (23). In our experience, inhibition was nullified by simple dilution of the sample. The reference techniques used for the comparative detection of microsporidia in stool specimens were Uvitex 2B and trichrome stainings. Our data indicated that PCR is as sensitive as those standard staining methods. In addition, it appears to be a detection system for the specific and accurate identification of intestinal microsporidia. Indeed, our PCR assay showed that *E. bieneusi* was exclusively present in four samples which were supposed to contain both species. This result confirms the restricted validity of the diagnosis of the specific infecting organism with conventional stains due to variabilities in spore size and shape between the species. In this situation, variations in *E. bieneusi* occurred. There was a good correlation between our IFA technique and PCR for the identification of *E. intestinalis*. Experiments with samples of isolated spores revealed a reproducible sensitivity of about 1 spore for *E. intestinalis* and 10 spores after Southern blot analysis for *E. bieneusi*. Such a difference indicates that the sensitivity of the assay depends on the quality of the tested sample. Whereas *E. intestinalis* was isolated from cultures, *E. bieneusi* was isolated from stools, and spores from stools are often damaged and frequently empty. In addition, the procedures required for flow cytometry analysis and sorting (Uvitex 2B staining and the intensity of the argon laser) might also cause damage. Furthermore, controls consisting of samples sorted by epifluorescence microscopy showed that spore

purity was not 100%; some fluorescent bacteria or same-size particles sorted together with microsporidian spores are a cause of the overestimation of the number of isolated spores.

The use of a digoxigenin-labelled internal probe for Southern hybridization has several advantages, the greatest of which is that it can be stored for a long time, unlike the commonly used ^{32}P-labelled probes. In addition, no radioactive material is used, so safety precautions and the problems of radioactive waste are avoided. In our experiments, the sensitivity of the digoxigenin-labelled probe was sufficient to confirm the presence of specific fragments obtained by agarose gel electrophoresis.

In addition to sensitivity and specificity, the economy in labor and time as well as the simplicity of the procedure will determine the usefulness of PCR as an assay for the detection of microsporidia. PCR will only work as a routine diagnostic tool when results are obtained within 1 or 2 days. Likewise, it can be used as an epidemiologic tool only when a large number of samples can be processed simultaneously. The boiling method contributes to the fulfillment of these conditions. Fecal samples used for PCR were prepared by the concentration method already used for the routine diagnosis of microsporidia in our laboratory. Thus, PCR can be combined with routine procedures, such as Uvitex 2B or Chromotrope 2R staining.

In conclusion, our results indicate that PCR is a valuable addition to the methods used to diagnose intestinal microsporidiosis. Preparation of the sample and performance of the assay can be done within 48 h, and the specificity of the assay with fecal specimens is high. These qualities indicate that our PCR assay may be the sensitive and rapid tool required for the diagnosis and determination of the epidemiology of microsporidioses.

ACKNOWLEDGMENTS

We are grateful to Pascal Petour, Nathalie Duval, Sandra Ktorza, Marie-Paule Nivez, Danièle de Nay, and Jean-Marc Bureau for technical assistance.

This study was supported by grants from SIDACTION and from Singer-Polignac Foundation.

REFERENCES

1. **Aldras, A. M., J. M. Orenstein, D. P. Kotler, J. A. Shadduck, and E. S. Didier.** 1994. Detection of microsporidia by indirect immunofluorescence antibody test using polyclonal and monoclonal antibodies. J. Clin. Microbiol. **32:**608–612.
2. **Asmuth, D. M., P. C. De Girolami, M. Federman, C. R. Ezratty, D. K. Pleskow, G. Desai, and C. A. Wanke.** 1994. Clinical features of microsporidiosis in patients with AIDS. Clin. Infect. Dis. **18:**819–825.
3. **Challier, S., S. Brown, C. Ombrouck, I. Desportes-Livage, D. De Nay, and M. Gentilini.** 1994. Flow cytometry as a possible method of isolation of spores of the microsporidian *Enterocytozoon bieneusi*. J. Eukaryot. Microbiol. **41:** 27S.
4. **Da Silva, A. J., D. A. Schwartz, G. S. Visvesvara, H. De Moura, S. B. Slemenda, and N. J. Pieniazek.** 1996. Sensitive PCR diagnosis of infections by *Enterocytozoon bieneusi* (microsporidia) using primers based on the region coding for small-subunit rRNA. J. Clin. Microbiol. **34:**986–987.
5. **Desportes, I., Y. Le Charpentier, A. Galian, F. Bernard, B. Cochand-Priollet, A. Lavergne, P. Ravisse, and R. Modigliani.** 1985. Occurrence of a new microsporidian, *Enterocytozoon bieneusi* n.g., n.sp., in the enterocytes of a human patient with AIDS. J. Protozool. **32:**250–254.
6. **Didier, E. S., J. M. Orenstein, A. Aldras, D. Bertucci, L. B. Rogers, and A. Janney.** 1995. Comparison of three staining methods for detecting microsporidia in fluids. J. Clin. Microbiol. **33:**3138–3145.
7. **Fedorko, D. P., N. A. Nelson, and C. P. Cartwright.** 1995. Identification of microsporidia in stool specimens by using PCR and restriction endonucleases. J. Clin. Microbiol. **33:**1739–1741.
8. **Franzen, C., A. Müller, P. Hegener, B. Salzberger, P. Hartmann, G. Fätenheuer, V. Diehl, and M. Schrappe.** 1995. Detection of microsporidia (*Enterocytozoon bieneusi*) in intestinal biopsy specimens from human immunodeficiency virus-infected patients by PCR. J. Clin. Microbiol. **33:**2294–2296.
9. **Hartskeerl, R. A., A. R. J. Schuitema, T. van Gool, and W. J. Terpstra.** 1993. Genetic evidence for the occurrence of extra-intestinal *Enterocytozoon bieneusi* infections. Nucleic Acids Res. **21:**4150.
10. **Hartskeerl, R. A., T. Van Gool, A. R. J. Schuitema, E. S. Didier, and W. J. Terpstra.** 1995. Genetic and immunological characterization of the microsporidian *Septata intestinalis* Cali, Kotler and Orenstein, 1993: reclassification to *Encephalitozoon intestinalis*. Parasitology **110:**277–285.
11. **Kokoskin, E., T. Gyorkos, A. Camus, L. Celidotte, T. Purtill, and B. Ward.** 1994. Modified technique for efficient detection of microsporidia. J. Clin. Microbiol. **32:**1074–1075.
12. **Kotler, D. P.** 1995. Gastrointestinal manifestations of immunodeficiency infection. Adv. Intern. Med. **40:**197–241.
13. **Ombrouck, C., I. Desportes-Livage, A. Achbarou, and M. Gentilini.** 1996. Specific detection of the microsporidia *Encephalitozoon intestinalis* in AIDS patients. C. R. Acad. Sci. **319:**39–43.
14. **Ombrouck, C., L. Ciceron, and I. Desportes-Livage.** 1996. Specific and rapid detection of microsporidia in stool specimens from AIDS patients by PCR. Parasite **3:**85–86.
15. **Orenstein, J. M.** 1991. Microsporidiosis in the acquired immunodeficiency syndrome. J. Parasitol. **77:**843–864.
16. **van Eys, G. J. J. M., C. Gravekamp, M. J. Gerritsen, W. Quint, M. T. E. Cornelissen, J. Ter Schegget, and W. J. Terpstra.** 1989. Detection of leptospires in urine by polymerase chain reaction. J. Clin. Microbiol. **27:**2258–2262.
17. **van Gool, T., E. U. Canning, H. Gilis, M. A. Van den Bergh Weerman, J. K. M. Eeftinck Schattenkerk, and J. Dankert.** 1994. *Septata intestinalis* frequently isolated from stool of AIDS patients with a new cultivation method. Parasitology **109:**281–289.
18. **van Gool, T., F. Snijders, J. K. M. Eeftinck-Schattenkerk, M. A. van den Bergh Weerman, J. F. W. M. Bartelsman, J. J. M. Bruins, E. U. Canning, and J. Dankert.** 1993. Diagnosis of intestinal and disseminated microsporidial infections in patients with HIV by a new rapid fluorescence technique. J. Clin. Pathol. **46:**694–699.
19. **Vavra, J., R. Dahbovia, W. S. Hollister, and E. U. Canning.** 1993. Staining of microsporidian spores by optical brighteners with remarks on the use of brighteners for the diagnosis of AIDS associated human microsporidioses. Folia Parasitol. **40:**267–272.
20. **Visvesvara, G. S., A. J. Da Silva, G. P. Croppo, N. J. Pieniazek, G. J. Leitch, D. Fergusson, H. De Moura, S. Wallace, S. B. Slemenda, I. Tyrrell, D. F. Moore, and J. Meador.** 1995. In vitro culture and serologic and molecular identification of *Septata intestinalis* isolated from urine of a patient with AIDS. J. Clin. Microbiol. **33:**930–936.
21. **Weber, R., R. T. Bryan, R. L. Owen, C. M. Wilcox, L. Gorelkin, and G. Visvesvara.** 1992. Improved light-microscopical detection of microsporidia spores in stool and duodenal aspirates. N. Engl. J. Med. **326:**161–166.
22. **Weiss, L. M., X. Zhu, A. Cali, H. B. Tanowitz, and M. Wittner.** 1994. Utility of microsporidian rRNA in diagnosis and phylogeny: a review. Folia Parasitol. **41:**81–90.
23. **Widjojoatmodjo, M. N., A. C. Fluit, R. Torensma, G. P. H. T. Verdonk, and J. Verhoef.** 1992. The magnetic immuno polymerase chain reaction assay for direct detection of salmonellae in fecal samples. J. Clin. Microbiol. **30:**3195–3199.
24. **Zhu, X., M. Wittner, H. B. Tanowitz, D. Kotler, A. Cali, and L. M. Weiss.** 1993. Small subunit rRNA sequence of *Enterocytozoon bieneusi* and its potential diagnostic role with use of the polymerase chain reaction. J. Infect. Dis. **168:**1570–1575.
25. **Zierdt, C. H., V. J. Gill, and W. S. Zierdt.** 1993. Detection of microsporidian spores in clinical samples by indirect fluorescent-antibody assay using whole-cell antisera to *Encephalitozoon cuniculi* and *Encephalitozoon hellem*. J. Clin. Microbiol. **31:**3071–3074.