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

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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 UVite2B 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 UVite2B 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 UVite2B 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 UVite 2B stain (18) and modified trichrome stain (11). Additionally, 42 stool samples examined for the presence of microsporidia by the UVite2B and Chromotrope 2R staining methods were coded and tested blind.
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 UVex 2B stain as described before (13).

Fecal samples were diluted in PBS with 10% Fomrol (vol/vol) and were filtered through a 50-μm pore-size filter. After centrifugation at 2,500 × g 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 at 10 min at 100°C by the method of van der Velde 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′-CACAGGGTGG ATTCGCGCTGAC-3′) and EB150 (5′-ACTACAGGTGTATACCTCAGCTC-3′) described by Zhu et al. (24) were used to amplify *E. bieneusi* DNA. The primers V1 and SI500 (5′-CTCCTCTCAATTACGCAA-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 mTris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl₂, 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, the amplification cycle consisted of an initial denaturation 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 radiolabeled probe.** 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′-GGGGTGCGCTAATGGGCTGCTGTCGTTGA-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-32P]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 × 10⁶ 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 scaled 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 labeled 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′-TGGGTATAGGTAAGGCTGCTGTCGTTGA-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 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.
with the \( ^{32}P \)-labelled EB150 probe, the assay could detect between 10 and 100 spores per 10 \( \mu \)l 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 microsporida, 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-IS500, 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 32P-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 samples used for PCR were prepared by the concentration of samples can be processed simultaneously. The boiling can be used as an epidemiologic tool only when a large number of microsporidia. PCR will only work as a routinediagnostic assay for the detection of microsporidia. The labor and time as well as the simplicity of the procedure will determine the usefulness of PCR as an efficientassay for the detection of microsporidia. J. Clin. Microbiol. 32:1074–1075.

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