

## Diagnosis of *Enterocytozoon bienersi* by PCR in Stool Samples Eluted from Filter Paper Disks

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**We report a PCR-based assay for the detection of *Enterocytozoon bienersi*. We extracted DNA from feces which had been applied to filter paper disks and evaluated four preserving solutions. Infected specimens were identified by electrophoresis of amplicons from concentrated formalin-fixed samples and unconcentrated fresh feces. Our findings demonstrate that this methodology is effective for sample collection, mailing, and diagnosis of this pathogen.**

The microsporidian species *Enterocytozoon bienersi* and *Encephalitozoon intestinalis* have been associated with chronic diarrhea and wasting in human immunodeficiency virus-infected patients (2, 12, 40). Diagnosis of gastrointestinal microsporidiosis depends on direct visualization of spores in stained fecal samples and species differentiation by transmission electron microscopy (6, 40, 41). Identification to the species level is clinically of great value because of the differences in therapy response (1, 13, 26). Most studies involve the use of PCR for detection of *E. bienersi* in fresh or formalin-fixed stool specimens (4, 7, 8, 9, 14, 15, 17–20, 28, 29, 33, 37, 38).

Epidemiological investigation of microsporidia has been limited by difficulties in the preservation and transport of feces and diagnostic methods applied to them. Samples collected on a filter paper are a valuable source of DNA for amplification-based methods and may provide a practical solution to many of these problems (3, 21, 30).

The purpose of this study was to describe a PCR assay for *E. bienersi* employing stool samples collected in different preserving solutions as impregnated filter paper disks (FPD).

Stool samples and intestinal biopsy specimens were obtained from three human immunodeficiency virus-infected patients with chronic diarrhea and microsporidiosis caused by *E. bienersi*. Microsporidial infections were diagnosed using multiple formalinized stool specimens by light-microscopic examination of samples stained according to Weber's modified trichrome method (40) and the quick-hot Gram-chromotrope technique (27). Five biopsy specimens from each patient were obtained from the distal duodenum by flexible fiber-optic endoscopy. Two specimens were fixed in 10% formalin for routine histology and were stained with Giemsa and hematoxylin-eosin. Two other specimens were processed for transmission electron microscopy (11, 31) to confirm *E. bienersi* infections. The fifth biopsy specimen was stored in saline solution at  $-20^{\circ}\text{C}$  for DNA purification. Stool samples were collected in four solutions: (i) 5% formaldehyde (volume ratio of stool to formalin, 1:3), (ii) 0.05% saline solution, (iii) 2.5% potassium dichromate, and (iv) Merthiolate-formalin (MF) (36) (volume ratio of stool to each of these three solutions, 1:2). FPD (Whatman no. 1) 1 cm in diameter were used to store the fecal specimens.

After homogenization, samples were spotted on the FPD in two different forms: as suspensions of homogeneous material and as centrifugation pellets following ethyl ether extraction (34). For pellet spotting, samples were resuspended in the remaining solution and 100  $\mu\text{l}$  was pipetted onto each disk. The FPD were air dried and stored in individual plastic bags at  $4^{\circ}\text{C}$  for at least 6 months.

Each FPD was incubated for 2 h at  $56^{\circ}\text{C}$  in 500  $\mu\text{l}$  of lysis buffer (100 mM Tris-HCl [pH 8.0], 100 mM EDTA [pH 8.0], 2% sodium dodecyl sulfate, 150 mM NaCl, and 200  $\mu\text{g}$  of proteinase K/ml). The DNA was purified by phenol-chloroform extractions and precipitation with absolute ethanol and was resuspended in 10  $\mu\text{l}$  of sterile redistilled water.

The primers Eb.gc (5'-TCAGTTTTGGGTGTGGTATCG G-3') and Eb.gt (5'-GCTACCCATACACACATCATTG-3') (38) were used to amplify a 210-bp fragment of the unique rRNA intergenic spacer sequence of *E. bienersi* (GenBank accession no. L20290) (43). All PCRs were carried out as previously described (38). Five microliters of DNA purified from each FPD was employed in this assay. The amplification procedure included 30 s of denaturation at  $98^{\circ}\text{C}$  in the first cycle ( $94^{\circ}\text{C}$  afterwards) followed by 30 s of annealing at  $49^{\circ}\text{C}$  and 90 s of extension at  $72^{\circ}\text{C}$  for 36 cycles. After the first PCR, 10  $\mu\text{l}$  of amplified material was used to run a second PCR under the same conditions and with the same primers as the first PCR. The amplified material (20  $\mu\text{l}$ ) was fractionated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. After the first round of PCR amplification, the analysis was performed on 12.5% polyacrylamide gels (22) followed by silver staining (PlusOne DNA silver stain kit; Pharmacia, Uppsala, Sweden).

The first round of PCR produced the expected DNA fragments on ethidium bromide- and silver-stained gels when DNA from concentrated fresh feces on FPD was used as a template (Fig. 1A). No bands were detected when DNA from uncentrifuged stool samples or specimens collected in preservative solutions was used. A second round of PCR resulted in efficient amplification of all non-formalin-fixed specimens, independent of the method used to apply feces to the membrane (Fig. 1B, lanes 1 to 4). Higher amounts of product from concentrated samples than from those placed on FPD as suspensions were observed for both fresh feces and potassium dichromate-preserved specimens. Amplicons of 210 bp could also be detected in the second round of PCR amplification using DNA

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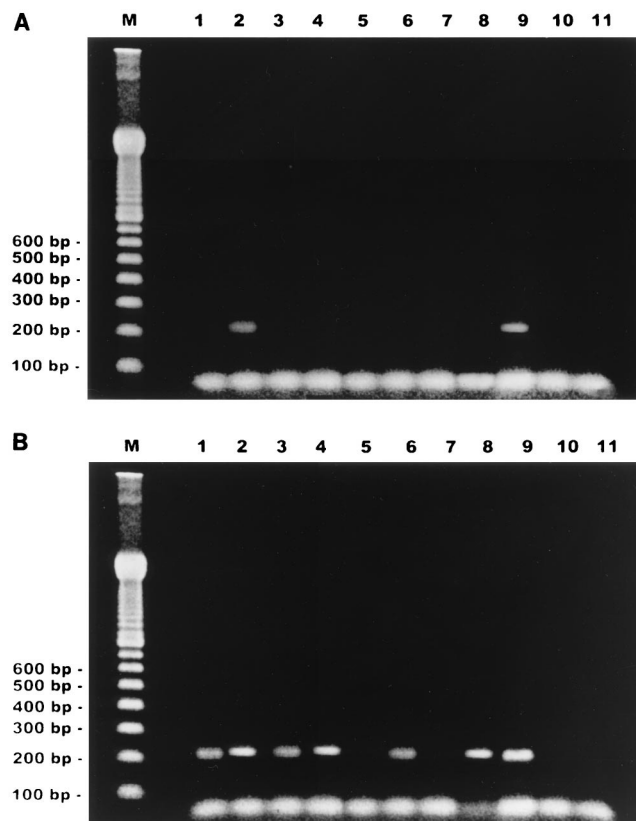


FIG. 1. Agarose gel electrophoresis (ethidium bromide stain) of PCR products. (A) First round of amplification; (B) double PCR amplification. Lanes 1 and 2, fresh sample; lanes 3 and 4, specimen in 2.5% potassium dichromate solution; lanes 5 and 6, formalin-fixed sample; lanes 7 and 8, specimen preserved in MF. Lanes 1, 3, 5, and 7, homogeneous suspension; lanes 2, 4, 6, and 8, concentrated sample. Lanes 9 and 10, positive and negative controls, respectively; lane 11, reaction mixture; lane M, 100-bp ladder.

prepared from formalin-fixed samples that had been ethyl ether extracted before FPD spotting (Fig. 1B, lane 6). No PCR products from nonconcentrated fixed specimens could be observed (Fig. 1B, lane 5). The same results were obtained with templates from samples collected in MF solution (Fig. 1B, lanes 7 and 8). Silver staining did not improve the results of the second PCR assay (data not shown). All results were identical for samples obtained from the three studied patients. DNA purified from frozen biopsy specimens of an *E. bienewsi*-infected patient was used as a positive control. DNA from duodenal biopsy specimens from known *E. bienewsi*-negative individuals was employed as a negative control in all assays.

The epidemiology of *E. bienewsi* infection has not been completely elucidated, and more information is needed on clinical features in HIV-infected patients and in other hosts, the impact of antiretroviral treatment, and geographical, environmental, and seasonal factors (5, 23, 40). Several PCR protocols for *E. bienewsi* have used fresh stool samples that were frozen or stored and transported at ambient temperatures for immediate processing (10, 32, 33, 37). Field conditions may limit the handling, transport, and refrigeration of the specimens. Other investigators have employed formalin-fixed fecal specimens, which can be transported and stored, but at present there is no information on the use of other fixatives for fecal specimens to be tested by microsporidial PCR protocols (4, 9, 14, 29).

In our study, examination of products after the first round of

PCR showed good amplification with DNA from centrifuged fresh feces spotted on FPD. Although some authors used different procedures to remove PCR inhibitors (15, 25, 36, 42), our results demonstrated that such removal was unnecessary with stool samples eluted from FPD. Negative results observed with preserved specimens showed a low level of DNA recovery. When fecal specimens are preserved in formalin, it is very difficult to amplify the DNA of microsporidial spores because this fixative reacts with the DNA (14). This possibility may be considered for the other fixatives employed (14, 16).

Our findings clearly show the advantage of our double PCR method and confirm that different preserved or fresh specimens can be employed with good diagnostic results. The filter paper specimens facilitate collection, transport, and storage because they have little weight, are cost effective, require minimal storage space, and maintain stable DNA during long periods (3, 24, 39). This study extends the diagnostic possibilities to laboratories employing all the usual collection procedures. Moreover, positive results obtained with unconcentrated fresh samples and the use of FPD as specimen support add great value to this technique because fecal specimens may be collected by untrained persons and mailed to a central diagnostic laboratory for identification.

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