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Sensitive PCR Diagnosis of Infections by *Enterocytozoon bieneusi* (Microsporidia) Using Primers Based on the Region Coding for Small-Subunit rRNA

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***Enterocytozoon bieneusi* is the most common microsporidian infecting patients with AIDS. We have developed a PCR primer pair, named EBIEF1/EBIER1, based on the small-subunit rRNA sequence of this microsporidian. Compared with other PCR-based methods, this primer pair shows a higher efficiency of detection in diagnostic applications than does another previously described primer pair, V1/EB450.**

Microsporidia are a group of obligatory parasitic amitochondrial protozoans that infect almost all animal phyla. In humans, microsporidia cause infections predominantly in immunocompromised patients. Among the five species of microsporidia involved in human infections, *Enterocytozoon bieneusi* is the most important, causing severe, long-lasting diarrhea, with a prevalence ranging from 7 to 50% (9). This wide range probably represents the low sensitivities of the methods used for the diagnosis of microsporidia, which is generally done by light or electron microscopy (1, 5, 6, 8).

PCR diagnosis of *E. bieneusi* was first reported by Zhu et al. (10), who used a generic microsporidian small-subunit rRNA (SSU-rRNA) forward primer (V1) and a reverse primer based on *E. bieneusi* SSU-rRNA (EB450). These primers were also used in other studies (3). We extensively tested the V1/EB450 primers and found that we did not get positive diagnostic results with some samples that were confirmed for the presence of *E. bieneusi* by careful electron microscopy evaluation. In addition, this set of primers also amplified DNA from another microsporidian (*Encephalitozoon hellem*). Thus, primer pair V1/EB450 is not specific and has to be used with caution. Moreover, we have resequenced the SSU-rRNA region for two geographically distinct isolates of *E. bieneusi* (7) and have shown that while our sequences were identical to the *E. bieneusi* SSU-rRNA sequence reported by Hartskeerl et al. (4), they showed 2.3% dissimilarity with the sequence reported by Zhu et al. (10), possibly because of sequencing errors in the latter sequence. When we examined the EB450 primer sequence, we found that it was based on a region where a nucleotide was possibly misread (position 9 of the EB450 primer has a G in place of a C). The resulting base mismatch for primer EB450, together with the use of a generic primer (V1) for which the *E. bieneusi* sequence is unknown, could definitely contribute to the low specificity and sensitivity of the V1/EB450 primer pair.

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In this report we describe a pair of highly specific PCR primers for diagnosis of *E. bieneusi* and compare them with primers V1/EB450. Our primers, named EBIEF1 and EBIER1, amplify a 607-bp DNA fragment. The forward primer, EBIEF1, 5′-GAAACTTGTCCACTCCTTACG-3′, and the reverse primer, EBIER1, 5′-CCATGCACCACTCCTGCCATT-3′, were based on nucleotides 295 to 315 and 881 to 901, respectively, of the *E. bieneusi* SSU-rRNA sequence (GenBank accession no. L16868 [4]). The optimal PCR conditions for the EBIEF1/EBIER1 primers were found to be denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s in a GenAmp 9600 PCR machine (Perkin-Elmer Cetus, Norwalk, Conn.). Concentration of each primer was 0.1 μg/50 μl of reaction mixture volume, which was prepared with the Perkin-Elmer Cetus GenAmp kit, according to the manufacturer's instructions.

First, the EBIEF1/EBIER1 PCR primer pair was tested for specificity with human DNA as well as with cloned microsporidian SSU-rRNA coding regions of 13 species of microsporidia, namely, *Encephalitozoon cuniculi*, *Encephalitozoon hellem*, *E. bieneusi*, *Enterocytozoon salmonis*, *Glugea atherinae*, *Nosema acridophagus*, *Nosema apis*, *Nosema furnacalis*, *Nosema necatrix*, *Nosema trichoplusiae*, *Pleistophora* sp. strain ATCC 50040, *Septata intestinalis*, and *Vittaforma corneae*. Positive amplification was only shown with the *E. bieneusi* cloned template, proving the high specificity of these primers (results not shown). Next, the sensitivity of the EBIEF1/EBIER1 primer pair was compared with that of the V1/EB450 primer pair. For the V1/EB450 primer pair, PCR conditions followed the recommendations of Zhu et al. (10), with the annealing temperature set at 50°C. The following samples were analyzed: a short-term in vitro culture of an *E. bieneusi* isolate, bile fluid from a patient with AIDS, and duodenal aspirate fixed in 100% ethanol for 24 h. All samples were confirmed for the presence of *E. bieneusi* by careful electron-microscopical evaluation. Samples were pelleted by centrifugation at 14,000 × *g* for 5 min and washed twice in phosphate-buffered saline, 0.01 M, pH 7.2, containing 1 mM EDTA, under the same conditions used for centrifugation. Pellets were suspended in 250 μl of the digestion buffer containing 50 mM Tris-HCl (pH 8.5), 1 mM EDTA, and 1% lauryl alcohol polyether (Laureth 12; PPG

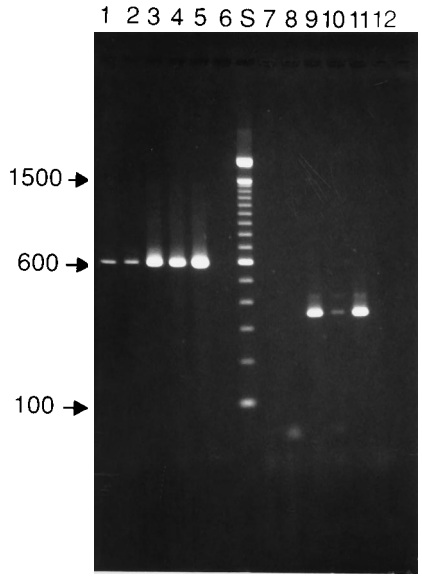

FIG. 1. Agarose gel analysis of PCR-amplified products. Lanes 1 to 6 show amplification with the EBIEF1/EBIER1 primer pair, and lanes 7 to 12 show amplification with the V1/EB450 primer pair. Analyzed samples: E6 cell culture infected with *E. bieneusi* (lanes 1 and 7); bile fluid from an AIDS patient (lanes 2 and 8); bile fluid, from an AIDS patient, further purified with the QIAquick PCR kit (lanes 3 and 9); duodenal aspirate biopsy sample from an AIDS patient (lanes 4 and 10); cloned *E. bieneusi* SSU-rRNA coding region (lanes 5 and 11). Lanes 6 and 12 contain negative controls (DNA isolated from uninfected E6 cells). Lane S contains the 100-bp ladder standard. Numbers to the left of the gel are DNA fragment sizes (in base pairs).

Industries Inc., Gurnee, Ill.). Proteinase K (Sigma Chemical Co., St. Louis, Mo.) was added to the concentration of 1 mg/ml, and this was followed by the addition of 0.1 g of glass beads (trimethylsilyl-silanized 140/270 mesh; Sigma) per 100 μl of total sample volume. Samples were agitated at full speed four times for 2.5 min, with 5 min of cooling on ice between bursts in a mini-bead beater (Biospec Products Inc., Bartlesville, Okla.). Next, samples were centrifuged at 14,000 × *g* for 20 s to pack the beads, and supernatants were transferred to clean tubes. Proteinase K was added again to the same concentration as described above, and samples were incubated overnight at 55°C. Finally, samples were incubated for 10 min at 95°C to inactivate proteinase K, allowed to cool at room temperature, and stored at 4°C until PCR amplification. In addition, an aliquot of the bile sample was concentrated before PCR with the QIAquick PCR kit (Qiagen, Chatsworth, Calif.) used per the manufacturer's instructions.

The results of PCR amplification clearly show the lower sensitivity of the V1/EB450 primer pair compared with that of the EBIEF1/EBIER1 primer pair. Only samples with high concentrations of the *E. bieneusi* target, namely, the QIA-quick PCR-concentrated bile sample, duodenal aspirate, and cloned *E. bieneusi* SSU-rRNA (Fig. 1, lanes 9, 10, and 11, respectively), were amplified with the V1/EB450 primers. In contrast, the EBIEF1/EBIER1 primers showed a positive result not only with the QIAquick PCR-concentrated bile sample, duodenal aspirate, and cloned *E. bieneusi* template, but also with the cultured sample and the unconcentrated bile sample (Fig. 1, lanes 1 and 2). In addition, primers EBIEF1/EBIER1 generated a stronger band for the duodenal aspirate than did primers V1/EB450 (Fig. 1, lanes 4 and 10).

These results clearly show the advantage of our primers and confirm that, for good diagnostic results, PCR primers have to be carefully designed with unique sequences free of sequencing artifacts. Further refinement of PCR diagnosis of *E. bieneusi* may involve the use of nested PCR or more sensitive detection techniques than ethidium bromide staining of PCR products after separation on an agarose gel. Besides being more sensitive, such methods are easier to perform in a diagnostic laboratory than methods for which PCR is followed by restriction enzyme digestion (2).

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