Parasitology

Multiplex detection of Enterocytozoon bieneusi and Encephalitozoon spp. in fecal samples using real-time PCR

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Abstract

A multiplex real-time polymerase chain reaction (PCR) method was developed for the simultaneous detection of Enterocytozoon bieneusi (n = 30) and Encephalitozoon spp. (n = 3) in stool samples. The multiplex PCR also included an internal control to detect inhibition of the amplification by fecal constituents in the sample. The assay was performed on species-specific DNA controls (n = 22) and a range of well-defined stool samples (n = 140), and it achieved 100% specificity and sensitivity. The use of this assay in a diagnostic laboratory offers the possibility of introducing DNA detection as a feasible technique in the routine diagnosis of intestinal microsporidian infections.

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1. Introduction

Microsporidia are small obligate intracellular parasites originally considered to be primitive eukaryotic protozoa but are recently reclassified with the fungi. Of the more than 1000 species of microsporidia, 14 species are known to infect humans of which Enterocytozoon bieneusi and Encephalitozoon intestinalis are responsible for most infections (Didier, 2005). E. bieneusi and E. intestinalis infections have been frequently recognized as a cause of severe diarrhea in persons with AIDS. Recently, intestinal microsporidiosis is increasingly diagnosed in other immunocompromised individuals such as organ transplant recipients, as well as in children, travelers, and elderly (Gomez Morales et al., 1995; Wanke et al., 1996; Gainzarain et al., 1998; Raynaud et al., 1998; Lopez-Velez et al., 1999; Muller et al., 2001; Lores et al., 2002; Leelayoova et al., 2005; Munthin et al., 2005; Wichro et al., 2005). Microscopic detection of the very small spores of microsporidia species in stool samples requires additional staining techniques, usually optical white stain (van Gool et al., 1994) or modified trichrome stain (Garcia, 2002). However, detection and differentiation of the spores from other fecal components after nonspecific optical white staining can still be difficult. Although the modified trichrome stain is more specific, interpretation of the slides can be very difficult because of the small size of the spores and the variability in the quality of the staining. Moreover, light microscopy does not allow accurate species determination, which is important, because treatment with albendazole is effective for E. intestinalis infection but not for E. bieneusi infections (Didier, 2005). An alternative drug, fumagillin, which is effective for both species (Didier, 2005), is also known to be very toxic and therefore not considered appropriate for the treatment of E. intestinalis infections.

Although polymerase chain reaction (PCR)-based methods have been successfully used for the detection of microsporidian infections (Katzwinkel-Wladarsch et al., 1996; Kock et al., 1997; Franzen and Muller, 1999; Notermans et al., 2005), their application in routine diagnosis is still very limited. Introduction of PCR-based methods has been hindered by difficulties in the DNA extraction from fecal samples. Moreover, the amplification and detection of DNA was prone to contamination as well as time consuming and expensive. In recent years, however, the isolation of parasitic DNA from fecal samples has been improved and simplified (Verweij et al., 2001; Subrungruang et al., 2004). The introduction of real-time PCR using fluorescent detection probes (Klein, 2002) can reduce
the risk of contamination, labor time, and reagent costs through the possibility of combining assays for the detection of different targets into one assay. Recently, real-time methods for the detection of Encephalitozoon spp. and E. bieneusi were published (Wolk et al., 2002; Menotti et al., 2003a, 2003b). However, because they were all separate assays without an internal control, the advantages of using real-time PCR were not fully exploited.

Therefore, a multiplex real-time PCR was developed for the simultaneous detection of E. bieneusi and Encephalitozoon spp. in fecal samples. In addition, an internal control for the detection of possible inhibition of the amplification by fecal contaminants was included in the assay. The performance of the assay was evaluated using a range of control samples.

2. Material and methods

2.1. Controls and samples

E. bieneusi control DNA was obtained from a human stool sample in which E. bieneusi was detected by fluorescence microscopy after optical white staining and confirmed by electron microscopy, and E. intestinalis control DNA was obtained from a cell culture of E. intestinalis originally isolated from human feces.

Thirty-three stool samples were selected in which microsporidian spores were detected by fluorescence microscopy after optical white staining, and E. bieneusi (n = 30) and Encephalitozoon spp. (n = 3) were confirmed by conventional PCR, which has been used routinely in our laboratory since 1996 (Katzwinkel-Wladarsch et al., 1996). All samples used were from different patients and collected from 1996 onward and stored at −20 °C (Table 1). Also, 60 unpreserved stool samples were tested from patients with a negative result in microscopy of formalin–ether sediments, modified acid-fast staining, and Giardia-antigen test. In these negative samples, 2 subsequent stool samples from these patients tested negative by all conventional methods.

Serial 10-fold dilution series of DNA extracted from each pathogen were tested with and without the presence of internal control DNA to estimate the influence of the internal control. Each dilution series was also tested with and without the other target to assess the ability to detect mixed infections.

Furthermore, DNA isolated from a serial dilution series of 100,000 E. bieneusi spores into negative feces was used to estimate the sensitivity of the PCR.

The specificity of the PCR was tested using Entamoeba histolytica, Entamoeba dispar, Giardia lamblia, Cryptosporidium parvum, or Cyclospora cayetanensis DNA as template. E. histolytica DNA was obtained from an axenic culture of E. histolytica HM1 strain, and E. dispar DNA from a polyxenic culture from human feces. G. lamblia DNA was isolated from purified G. lamblia cysts, and C. parvum DNA from purified C. parvum oocysts (Waterborne, New Orleans, LA). C. cayetanensis DNA was isolated from a human stool sample known to contain C. cayetanensis oocysts and confirmed with C. cayetanensis-specific real-time PCR (Verweij et al., 2003a). Specificity of the assay was also tested on DNA obtained from 17 different bacterial/yeast cultures: Bacillus cereus, Campylobacter jejuni, Campylobacter upsaliensis, Candida albicans, Escherichia coli O157, Enterobacter aerogenes, Enterococcus faecalis, Proteus mirabilis, Pseudomonas aerogenosa, Salmonella enteritis, Salmonella typhimurium, Shigella boydii, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Staphylococcus aureus, and Yersinia enterocolitica. All isolates were grown and typed biochemically using routine bacteriologic procedures.

Furthermore, 80 stool samples from different patients were tested in which E. histolytica (n = 20), E. dispar (n = 20), G. lamblia (n = 20), or C. parvum/Cryptosporidium hominis (n = 20) was detected by microscopy and confirmed by specific PCR, respectively (Verweij et al., 2003b, 2004).

2.2. Microscopy

Microscopic examination for the presence of ova and cysts was performed routinely by examination of iodine stained wet mount after formal-ether concentration using magnification ×400. Modified acid-fast staining was performed on direct smears and formal-ether concentrates for detection of coccidian parasites. Fluorescence microscopy after optical white staining was performed on direct smears and saline–ether concentrates for the detection of microsporidian spores (van Gool et al., 1994).

2.3. DNA isolation

For DNA isolation, 200 µL of feces suspension (≈1 g/mL of phosphate-buffered saline containing 2% polyvinylpolypyrrolidone, Sigma-Aldrich, Zwijndrecht, The Netherlands) was heated for 10 min at 100 °C. After sodium dodecyl sulfate–proteinase K treatment (2 h at 55 °C), DNA was isolated with the QIAamp Tissue Kit spin columns (QIAGen, Hilden, Germany) (Verweij et al., 2001). If formalin-fixed fecal samples are used, the formalin has to be removed by washing/centrifuging steps with saline direct or after formalin–ether concentration. However, sensitivity of amplification is known to decrease with time of fixation.
In each sample, 10^3 PFU/mL phocine herpes virus 1 (PhHV-1) was added within the isolation lysis buffer to serve as an internal extraction and amplification control (Niesters, 2002).

2.4. PCR amplification and detection

*E. bieneusi*-specific PCR primers and a detection probe were chosen using Primer Express software (Applied Biosystems, Foster City, CA) on the internal transcribed spacer (ITS) sequence for *E. bieneusi* (GenBank accession no. AF101198) such that a 103-bp fragment within the ITS sequence should be amplified and detected for *E. bieneusi* specifically. The *E. bieneusi*-specific primers and probe set consisted of forward primer EbITS-89F, reverse primer EbITS-191R, and *E. bieneusi*-specific double-labeled probe EbITS-114revT (Eurogentec, Belgium).

*Encephalitozoon* spp. PCR primers and detection probe were designed using Primer Express software (Applied Biosystems) on the small subunit ribosomal RNA gene sequence for *E. intestinalis* (GenBank accession no. U09929) such that *Encephalitozoon* spp. DNA would be specifically amplified and detected. The forward primer MSP1F and reverse primer Eint213R amplified a 214-bp fragment inside the SSU rRNA gene. The *Encephalitozoon* spp.-specific double-labeled probe Eint82Trev (Eurogentec) was used to detect *Encephalitozoon* spp.-specific amplification.

PhHV-1 specific primers and probe (Niesters, 2002) set consisted of forward primer PhHV-267s, reverse primer PhHV-337as, and *E. bieneusi*-specific double-labeled probe PhHV-305tq (Biolegio, The Netherlands).

Amplification reactions were performed in a volume of 25 μL with PCR buffer (HotstarTaq master mix, QIAgen), 5 mM MgCl₂, 2 pmol of each *E. bieneusi*-specific primer, 6.25 pmol of each *E. intestinalis*-specific primer, 3.75 pmol of each PhHV-1-specific primer, 2.5 pmol of *Encephalitozoon* spp.-specific double-labeled probe, 2.5 pmol of PhHV-1-specific double-labeled probe, and 5 μL of the DNA sample. Amplification consisted of 15 min at 95 °C followed by 50 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Amplification, detection, and data analysis were performed with the I-cycler real-time detection system (BioRad, Hercules, CA). Fluorescence of FAM, Yakima Yellow, and CY5 was measured at their respective wavelengths during the annealing step of each cycle. All primers and detection probes are described in Table 2.

3. Results

The specificity of the real-time multiplex PCR was evaluated using a range of controls: DNA from *E. histolytica*, *E. dispar*, *G. lamblia*, *C. parvum*, or *C. cayetanensis*, and from *B. cereus*, *C. jejuni*, *C. upsaliensis*, *C. albicans*, *E. coli* O157, *E. aerogenes*, *E. faecalis*, *P. mirabilis*, *P. aerogenosa*, *S. enteritidis*, *S. typhimurium*, *B. boydii*, *S. dysenteriae*, *S. flexneri*, *S. sonnei*, *S. aureus*, and *Y. enterocolitica*. In none of these samples amplification was detected with either of the 2 assays. Furthermore, 80 DNA extracts derived from feces positive for *E. histolytica* (n = 20), *E. dispar* (n = 20), *G. lamblia* (n = 20), or *C. parvum* (n = 20), and 60 DNA extracts derived from feces from individuals with no known history of parasitic infections were tested. No amplification was detected of *E. bieneusi* or *Encephalitozoon* spp. DNA in any of these samples; only the amplification of the internal control was detected at the expected threshold cycle of approximately 33.

The cycle threshold (Ct) values obtained from testing the dilution series of each pathogen in the individual assay and the multiplex assay were similar, and the same analytical sensitivity was achieved. Thus, individual performance of the assays was not influenced by the presence of DNA from the internal control or by the presence of DNA from the other targets.

One *E. bieneusi* spore was estimated as the detection limit of the isolation and PCR procedure.

*E. bieneusi*-specific amplification was detected in 30 of 33 DNA samples isolated from feces known to contain microsporidian spores. Threshold cycles (Ct values) were found between 15.8 and 36.5 (Table 2) with a median threshold of 21.6 cycles. *Encephalitozoon* spp.-specific amplification was detected in the other 3 samples in which microsporidian spores were detected by microscopic examination, with Ct values of 22.4, 24.7, and 34.0, respectively.
4. Discussion

The use of labor-intensive additional staining techniques for the microscopic diagnosis of microsporidia infections will often be limited to samples of severe immune-suppressed patients. Many laboratories will not see as many of these patients because of the success of highly active antiretroviral therapy (HAART) therapy in HIV-infected individuals. As a consequence, gaining expertise is difficult because positive findings will be scarce. Therefore, many infections in patients under relatively mild immune-suppressed conditions and immunocompetent patients will probably stay undiagnosed. The multiplex real-time PCR for the detection of *E. bieneusi* and *Encephalitozoon* spp. presented in this study gives a useful alternative for the labor-intensive and expertise-dependent microscopic methods.

Using well-defined DNA and stool samples as control, the multiplex real-time assay for the detection of *E. bieneusi* and *Encephalitozoon* spp. achieved specificity of 100%. In all samples tested in which microscopy revealed the presence of microsporidian spores, specific amplification was detected for *E. bieneusi* (n = 30) or *Encephalitozoon* spp. (n = 3). There was no difference in the performance of the amplification of the specific targets in the individual assays as compared with the multiplex PCR so the multiplex PCR could be used with equal confidence to the individual assays. PCR inhibition by fecal constituents is known to be a serious problem (Monteiro et al., 1997). However, in stool samples without a known history of parasitic infection and in stool samples with *E. histolytica*, *E. dispar*, *G. lamblia*, or *C. parvum/C. hominis*, only the amplification of the internal control was detected. Hence, there was no evidence of inhibition of the amplification in any of these samples using this DNA isolation method.

The *Encephalitozoon* spp. primers and probe were designed on the known sequence of *E. intestinalis*. According to NCBI BLAST, however, also *Encephalitozoon hellem* and *Encephalitozoon cuniculi* will be amplified and detected with this primer/probe combination. Although determination to the genus level will be sufficient in clinical practice, species-specific differentiation can be achieved by sequencing of the PCR product.

*E. intestinalis* is also known to cause extraintestinal disease such as infections of the pulmonary and urinary tract, as well as disseminated infections. Although we have not focused on the detection of microsporidium in nonintestinal samples, we expect that the described PCR can also be used on these samples. Laboratories that have this specific diagnostic question would have to use their own validated protocols for the isolation of parasitic DNA from these samples and use this DNA in the described PCR.

Detection of parasite DNA has the potential to be more sensitive as compared with microscopy; this has already been shown for *G. lamblia*, *E. histolytica/E. dispar*, *C. parvum/C. hominis*, and *E. bieneusi* using (real-time) PCR (Webster et al., 1996; Morgan et al., 1998; Ghosh et al., 2000; Menotti et al., 2003a; Verweij et al., 2003b, 2003c, 2004). Considering the estimated detection limit of 1 spore in the PCR and the low Ct values obtained in these microscopy-positive samples, this multiplex real-time PCR for the detection of *E. bieneusi* and *Encephalitozoon* spp. certainly has the potential to detect microsporidia infections in patients shedding very low numbers of spores.

Previously, we already postulated on the tremendous impact the implementation of automated DNA isolation procedures and other multiplex assays for the detection of parasites, viruses, and bacteria that cause diarrhea will have on the differential laboratory diagnosis of diarrheal diseases (Verweij et al., 2004). The multiplex real-time PCR described here is a sensitive and specific method for the detection of *E. bieneusi* and *Encephalitozoon* spp. and offers the possibility of introducing DNA detection as a feasible technique in the routine diagnosis of intestinal microsporidial infections and can also be used as a part of a series of other multiplex assays in the differential diagnosis of diarrhea-causing pathogens.

References


