Identification of *Encephalitozoon intestinalis* in Travelers with Chronic Diarrhea by Specific PCR Amplification

LAURENT RAYNAUD,1,2 FREDERIC DELBAC,3 VERONIQUE BROUSSOLLE,3 MEJA RABODONIRINA,2 VERONIQUE GIRault,2 MARTINE WALLON,2 GREGOIRE COZON,4 CHRISTIAN P. VIVARES,3 AND FRANCOIS PEYRON,2*

ESSA, 69500 Bron,1 Département de Parasitologie, Faculté de Médecine, Université Claude Bernard, 69373 Lyon Cedex 08,2 Protistologie Moléculaire et Cellulaire des Parasites Opportunistes, LBCP, ESA CNRS 6023, Université Blaise Pascal, 63177 Aubière Cedex,2 and Service Immunologie, Hôpital de la Croix Rousse, 69317 Lyon Cedex 04,4 France

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With the use of Weber’s modified trichrome and Uvitex 2B techniques, spores of microsporidia were detected in the stools of four travelers presenting with chronic diarrhea. The general health of these patients was not impaired, and human immunodeficiency virus screening was negative. Immune evaluation, including the study of lymphocytic subpopulations, assay of serum immunoglobulins, and an intradermal multitest, showed normal results. Molecular identification of microsporidian species was based on the PCR amplification of a small-subunit rRNA sequence followed by *HinI* endonuclease restriction. *Encephalitozoon intestinalis* microsporidiosis was thus shown in two of the four patients examined. In two patients, therapy based on albendazole made stools devoid of microsporidian spores without influence on the intestinal disorders. The pathogenic role of *E. intestinalis* in immunocompetent individuals remains to be demonstrated.

Microsporidia are ubiquitous intracellular parasitic protozoa affecting the whole animal kingdom (4). Seven genera are pathogenic in humans: *Encephalitozoon*, *Enteroctozoon*, *Nosema*, *Pleistophora*, *Vittaforma*, *Trachypleistophora*, and *Microsporidium*, the latter including all species with undetermined status. Some of them are known to be opportunistic pathogens in immunodepressed patients (8). They may cause chronic diarrhea with food malabsorption, as well as disseminated impairments (2, 3, 7). Ocular and gastrointestinal failures related to microsporidia have also been described in immunocompetent ones (1, 5, 10, 14, 17, 18). Staining methods (19, 22) and immunodiagnostic tests (15, 20, 21, 23, 24) can differentiate ones (1, 5, 10, 14, 17, 18). Staining methods (19, 22) and immunodiagnostic tests (15, 20, 21, 23, 24) can differentiate microsporidia from bacteria and yeasts in clinical samples such as stool samples, but precise identification of the species involved is not always successful. Recently, PCR amplification of conserved ribosomal DNA (rDNA) sequences was used to detect intestinal microsporidia in biopsy and stool specimens (6, 9, 11, 13, 16).

We screened four human immunodeficiency virus (HIV)-negative travelers with chronic diarrhea for microsporidia and gave them a complete immunologic evaluation. After detection of spores in stool samples by light microscopy, we tried to identify the corresponding parasite species. However, electron microscopy failed to provide evidence of microsporidia and no significant results were obtained for the detection of *Encephalitozoon cuniculi* by Western blotting and enzyme-linked immunosorbent assay. Thus, we decided to develop a new PCR procedure ensuring the differentiation of any known microsporidian species pathogenic to humans. This procedure enabled us to identify *Encephalitozoon intestinalis* in two immunocompetent patients.

*Corresponding author. Mailing address: Département de Parasitologie, Faculté de Médecine, Université Claude Bernard, 8 Avenue Rockefeller 69373 Lyon Cedex 08, France. Phone: 33-04-78-77-70-00. Fax: 0033-04-78-75-17-72. E-mail: peyron@csismun.univ-lyon1.fr.

**MATERIALS AND METHODS**

**Patients.** The patients were travelers presenting with chronic diarrhea. When microsporidian spores were detected in stool samples as judged by Weber’s modified trichrome (22) and Uvitex 2B (19) techniques, an immunologic evaluation was performed including HIV tests, assay of immunoglobulins, and a study of lymphocytic subpopulations by flow cytometry after triple labeling with anti-CD3, -CD4, -CD8, -CD56 (NK cells), and -DC19 (B lymphocytes) antibodies, and an intradermal multitest (Bio-Mérieux, Marcy l’Etoile, France). Treatment with albendazole 400 mg twice daily was prescribed for 20 days, and the patients were re-examined 1 month after the end of the treatment.

**Clinical samples from two homosexual AIDS patients (CD4+ cells per μl) with intestinal microsporidiosis were used as a positive control.**

**Stool samples and parasite cultures.** Formalin-fixed stool samples were washed several times in phosphate-buffered saline (PBS) and stored at 4°C. *Septata intestinalis* Cali, Kotler, and Orenstein 1992 (3), subsequently reclassified as *Encephalitozoon intestinalis* by Hartskerel et al. (12); *E. cuniculi*; and *E. hellem* were grown in vitro in MRC-5 human lung fibroblasts (Bio-Mérieux) or Madin-Darby canine kidney (MDCK) cells (Bio-Whittaker) in 75-cm² tissue culture flasks (Polylabo) containing minimum essential medium supplemented with 1-glutamine, 5% fetal calf serum, and diverse antibiotics (ampicillin, penicillin, and streptomycin). The cell cultures were incubated at 37°C with 5% CO₂ in an air atmosphere. Supernatants containing mature spores were collected; spores were then sedimented by centrifugation, washed, and stored in 0.1 M PBS (pH 7.4) at 4°C.

**DNA extraction.** Stool specimens were mixed with 1 volume of PBS buffer and then centrifuged at 15,000 × g for 2 min. The pellets were washed in PBS and resuspended in 1 ml of 1% sodium dodecyl sulfate–300 mM Tris (pH 9.0)–100 mM EDTA. After incubation at 65°C for 30 min, suspensions were centrifuged and resuspended in 500 μl of lysis buffer (10 mM Tris, 100 mM NaCl, 1 mg of proteinase K [Sigma] per ml, 200 U of Lyticase [Sigma]). Mechanical disruption was performed with zirconium beads (0.1-mm diameter; Biospec Products Inc., Bartlesville, Okla.). Following addition of 2% sodium dodecyl sulfate and 1 mg of proteinase K per ml, extracts were incubated at 55°C for 3 h and then proteins were precipitated with 1 M potassium acetate for 1 h at 4°C. DNA was phenol-chloroform extracted, precipitated with ethanol for 1 h, and resuspended in 50 μl of sterile water.

*E. cuniculi*, *E. hellem*, and *E. intestinalis* spores collected from MRC-5 or MDCK cell cultures were boiled at 100°C for 10 min to release DNA.

**PCR amplification.** Primers for PCR were chosen to amplify a conserved region of the small-subunit (SSU) rRNA gene of four microsporidia reported in AIDS patients: *E. cuniculi*, *E. hellem*, *E. intestinalis*, and *Enteroctozoon bieneusi*. Forward primer C1 (5′-CACCAAGTGGATTCATGCC-3′) and reverse primer C2 (5′-CTGACGGCGGCTGTAC-3′) were determined by GenBank sequence analysis of these species. C1 was complementary to bases 1 to 18 of each one, C2

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sequences deposited in GenBank, it could be predicted that these species may be differentiated on the basis of the number of HinIII restriction sites: one for \textit{E. cuniculi}, two for \textit{E. hellem}, and three for \textit{E. intestinalis}, excluding one site at position 10 within each amplicon. Species-specific restriction patterns were indeed observed, the sizes of the different fragments being 350 and 830 bp for \textit{E. cuniculi} (Fig. 3, lane 2); 120, 250, 350, and 460 for \textit{E. intestinalis} (lane 3); and 260, 350, and 580 for \textit{E. hellem} (lane 4). Digestion with the same enzyme of 1,200-bp amplicons from the two AIDS patients produced two distinctive DNA fragments with sizes (230 and 940 bp; Fig. 3, lanes 7 and 8) different from those of \textit{E. cuniculi} (350 and 830 bp). It was confirmed that the two immunocompromised patients were infected with \textit{E. bieneusi}, the amplified rDNA sequence of which displays one HinIII restriction site, at position 238.

\section*{Diagnosis of diarrheic travelers}
For this study, four travelers were selected. They were males with a mean age of 29 years and had had diarrhea for 1 to 71 months. They had traveled in Africa, Nepal, or Southeast Asia. The mean daily number of stools was four, they contained neither blood nor mucus, and

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1}
\caption{Analysis of PCR products by 1.5\% agarose gel electrophoresis. A 1,200-bp DNA fragment of the SSU rDNA gene of microsporidia was amplified by using primers C1 and C2. Molecular size markers (lane m) were \lambda phage DNA digested with EcoRI and HindIII. DNA extracts were obtained from \textit{E. intestinalis} in MRC-5 cell cultures (lane 1, positive control), immunocompetent patient (IC1, IC2, IC3, and IC4) stools (lanes 2, 3, 4, and 5), HIV-infected patient (SID1 and SID2) stools (lanes 6 and 7), and a negative control (lane 8).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Digestion of PCR products (1,200-bp DNA fragment) by HindIII restriction endonuclease. Shown is a 1.5\% agarose gel containing \textit{E. intestinalis} rDNA amplified before (lane 1) and after (lane 2) HindIII digestion and digested, amplified fragments from patients IC1 and IC3 (lanes 3 and 4) and patients SID1 and SID2 (lanes 5 and 6). Lane 7 contained a negative control. Two bands were obtained from \textit{E. bieneusi}-infected patients (800 and 400 bp). Amplified DNA fragments of \textit{E. intestinalis}, \textit{E. cuniculi}, and \textit{E. hellem} were not cleaved by HindIII. Lane m contained the same molecular size markers as lane m in Fig. 1.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Digestion of a 1,200-bp amplicon by restriction endonuclease HindIII. Shown is a 2\% agarose gel. Lanes: 1, 1,200-bp PCR product before HindIII digestion; 2, \textit{E. cuniculi}; 3, \textit{E. intestinalis}; lane 4, \textit{E. hellem}; 5 and 6, patients IC1 and IC3; 7 and 8, patients SID1 and SID2. Lane m contained the same molecular size markers as lane m in Fig. 1.}
\end{figure}
the patients’ general health was good. Only one patient com-
plained of nonsystematic abdominal pain. The four travelers (IC1, IC2, IC3, and IC4) were HIV negative. The test for
lymphocytic subpopulations and the cutaneous multitest yield-
ed normal results (Table 1). Immunoglobulin levels in serum
were normal, except in the patient with protracted diarrhea (6
years), whose immunoglobulin A (IgA) concentration was 0.86
g/liter (normal, 1 to 3 g/liter). An assay of the IgA in the saliva
of this patient yielded 100 mg/liter (normal range, 87 to 500
mg/liter). Stool examinations using Weber’s modified tri-
chrome and Uvitex 2B techniques revealed the presence of
microsporidial spores. The patients were also screened for
parasites by direct examination, culture of stools, and a Ziehl-
Neelsen test for cryptosporidia, and no evidence of eukaryotic
pathogens other than microsporidia was obtained.

The above-described PCR amplification procedure was ap-
plicated to stool samples from four nonimmunocompromised pa-
tients. A positive response was obtained with samples from
only patients IC1 and IC3 (Fig. 1, lanes 2 and 4), suggesting a
microsporidial infection. HinfI digestion showed the four
bands which are relatable to the presence of E. intestinalis (Fig.
3, lanes 5 and 6). The stool samples from patients IC2 and IC4
were spiked with cultured E. cuniculi spores. The expected
amplified products were obtained, the detection limit of par-
ticular samples varying between 20 and 100 spores per 0.1 g of
stool. Repeated sampling provided the same results.

**DISCUSSION**

Our PCR method involves the digestion of an amplified
product as described by Fedorko et al. (9), except that the
product corresponds to the much larger fragment of the SSU
rRNA gene (more than 90% of the whole length). Ombrouck
et al. (16) have detected E. intestinalis in stool samples from
AIDS patients by amplifying a 380-bp DNA fragment with a
specific reverse primer. Since it has been recently shown that
E. cuniculi and E. hellem spores could be present in stools of
AIDS patients (6), the differentiation of Encephalitozoon spe-
cies is essential. This has been achieved through HinfI diges-
tion of the 1,200-bp ampiclon, which provides specific banding
patterns. E. bieneusi and the three Encephalitozoon species can
be specifically identified. The data support an E. intestinalis
infection in two nonimmunocompromised humans (IC1 and
IC3). Our DNA extraction and analysis protocol for stool sam-
pies is realizable in 1 day. This time could be shortened if a
heating procedure were used for cell lysis (16).

Microsporidial pathogenicity in immunocompetent patients
is still poorly understood, and difficulties in diagnosis persist.
In the patients examined here, the Uvitex 2B and Weber stain-
ing methods (19, 22) provided evidence of microsporidian in-
festation independent of the HIV syndrome. An IgA deficit can
be ruled out in our patients. This deficit occurs frequently, and

**TABLE 1. Immunological characteristics of patients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>No. of cells/μl of blood (%)</th>
<th>CD4/CD8 cell ratio</th>
<th>Concn (g/liter of blood) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4⁺</td>
<td>CD8⁺</td>
<td>NK</td>
</tr>
<tr>
<td>IC1</td>
<td>849 (42)</td>
<td>667 (33)</td>
<td>202 (10)</td>
</tr>
<tr>
<td>IC2</td>
<td>968 (53)</td>
<td>383 (21)</td>
<td>201 (11)</td>
</tr>
<tr>
<td>IC3</td>
<td>858 (39)</td>
<td>440 (20)</td>
<td>308 (14)</td>
</tr>
<tr>
<td>IC4</td>
<td>538 (47)</td>
<td>217 (19)</td>
<td>103 (9)</td>
</tr>
</tbody>
</table>

Normal range: 500–1,700, 250–1,200, <600 (<25), 100–400, 0.5–2, 1–3, 7–13, 0.6–2.

* BL, B lymphocyte.

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