

Detection of Microsporidia in Travelers with Diarrhea

A. MÜLLER,¹ R. BIALEK,² A. KÄMPER,³ G. FÄTKENHEUER,³ B. SALZBERGER,³ AND C. FRANZEN^{3*}

Department of Pediatrics¹ and Department of Internal Medicine I,³ University of Cologne, Cologne, and Institut for Tropical Medicine, University of Tübingen, Tübingen,² Germany

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We examined stool specimens of 148 returning travelers from an outpatient department for tropical diseases for the appearance of microsporidia using light microscopy and PCR. Intestinal microsporidiosis was diagnosed for five patients by light microscopy and for nine patients by PCR. Some cases were diagnosed only by PCR, indicating that the true prevalence has to be determined by highly sensitive techniques, such as PCR.

Diarrhea affects 20 to 70% of travelers in tropical or subtropical countries. Typically, self-limiting travelers' diarrhea is more of an inconvenience than a life-threatening disease. In general, bacteria, e.g., *Escherichia coli*, are the most common pathogens isolated in travelers' diarrhea. However, different intestinal protozoan parasites are recognized in travelers with diarrhea as well. Microsporidia are important protozoan parasites in human immunodeficiency virus (HIV)-infected patients. Limited data are available about microsporidian infections in immunocompetent patients and travelers (1, 17, 19, 21).

Stool samples were obtained from 148 travelers with diarrhea returning from different tropical areas between January and March 1998. Stool samples were examined by light microscopy (Uvitex 2B staining) and PCR as previously described (16). PCR products were digested with the restriction endonucleases *Pst*I (20 U) and *Hae*III (10 U) (Sigma) in a final volume of 15 μ l, producing distinctive fragments. The 250-bp DNA fragment of *Enterocytozoon bienersi* was not cut by *Pst*I and produced a 208-bp DNA fragment after digestion with *Hae*III. The 268-bp DNA fragment of *Encephalitozoon cuniculi* was cut by *Pst*I into two, 122- and 146-bp DNA fragments and by *Hae*III into a 189-bp DNA fragment. The 270-bp DNA fragment of *Encephalitozoon intestinalis* was cut by *Pst*I into two, 124- and 146-bp DNA fragments and by *Hae*III into a 193-bp DNA fragment. The 279-bp DNA fragment of *Encephalitozoon hellem* was cut by *Pst*I into two, 133- and 146-bp DNA fragments and by *Hae*III into a 200-bp DNA fragment (9). Purified DNA fragments were ligated into a pGEM-T vector (Promega). Plasmids were transfected in competent *E. coli* cells (JM109) and plated on ampicillin-isopropyl- β -D-thiogalactopyranoside-S-bromo-4-chloro-3-indolyl- β -D-galactopyranoside agar plates for blue-white screening. White colonies were selected, and plasmids were isolated from the cells by using the High Pure plasmid isolation kit (Boehringer, Mannheim, Germany). Both DNA strands of the cloned DNA fragments were sequenced on an automated DNA sequencer (ABI Prism 377 DNA Sequencer; Applied Biosystems) using the *Taq* FS BigDye-terminator cycle sequencing method starting with

two vector-directed primers (T7 primer [5'-TAATACGACTC ACTATAGGG-3'] and M-13 reverse primer [5'-TTCACACA GGAAACAGCTATGACG-3']) flanking the cloned DNA fragments.

Examination of stool samples from 148 returning travelers by light microscopy showed microsporidian spores in stool samples of five patients. One case was identified as *E. bienersi* because of the small size of the spores (2 μ m). In all other cases, species differentiation was not possible. PCR amplification produced 250-bp DNA fragments, indicating infection with *E. bienersi*, from stool specimens of seven patients and from plasmids with the small-subunit (SSU) rRNA sequence of *E. bienersi*. Four of these patients were negative by light microscopy (Table 1). An approximately 270- to 280-bp DNA fragment, indicating infection with an *Encephalitozoon* spp., was amplified from stool specimens of four patients and from plasmids containing the SSU-rRNA genes of *E. intestinalis*, *E. hellem*, and *E. cuniculi* (Table 1). In two specimens, 250-bp and 270- to 280-bp DNA fragments were amplified. Results of digestion with the restriction endonucleases *Pst*I and *Hae*III and of DNA sequencing are shown in Table 1. This confirms infection with *E. bienersi* in five cases and double infection with *E. bienersi* and *E. hellem* in two cases.

New pathogens continue to be discovered as the cause of travelers' diarrhea, and a few case reports have demonstrated that microsporidia may be the cause of travelers' diarrhea (1, 17, 19, 21). Human microsporidiosis has been reported predominantly from developed nations in North America, Europe, and Australia, but cases are now increasingly identified in the developing countries as well, and human infections with microsporidia have been reported from several African nations (2, 4, 6, 7, 11, 12, 14, 22), Southeast Asia (15), and Central and South America (3, 8, 24, 25). Although reliable estimates of the prevalence of microsporidiosis in developing countries are not available, some studies seem to indicate that the prevalence of intestinal microsporidiosis may be high in developing countries (10, 22). Most cases of human microsporidiosis are associated with immunosuppression, but increasing numbers of cases in non-HIV-infected immunocompetent patients are reported. These reports include infections of travelers to developing countries as well as infections of residents of various tropical countries (1, 5, 6, 11, 13, 14, 17, 19–21, 23). Only a few studies examined the role of microsporidia in returning travelers. One study examined stool samples of 750 Swedish travelers with

* Corresponding author. Mailing address: Department of Internal Medicine I, University of Cologne, Joseph-Stelzmann Str. 9, D-50924 Cologne, Germany. Phone: 49-(0)221-478-4433. Fax: 49-(0)221-478-5915. E-mail: Caspar.Franzen@Uni-Koeln.de.

TABLE 1. Results of light microscopy, PCR amplification, digestion of PCR products, and DNA sequencing of amplified PCR products from nine patients with microsporidiosis

Patient no.	Microsporidian species	Detection of spores by light microscopy	Length (bp) of DNA fragment(s)			% Homology ^b
			After PCR ^a	After digestion with:		
				<i>Pst</i> I	<i>Hae</i> III	
1	<i>E. bienewsi</i>	+	250	250	208	99.2 ^d
2	<i>E. bienewsi</i>	+	250	250	208	99.6 ^d
3	<i>E. bienewsi</i>	+	250	250	208	99.6 ^d
4	<i>E. bienewsi</i>	–	250	250	208	99.6 ^d
5	<i>E. bienewsi</i>	–	250	250	208	ND
6	<i>Encephalitozoon</i> sp.	+	270	ND ^c	ND	ND
7	<i>Encephalitozoon</i> sp.	+	270	ND	ND	ND
8	<i>E. bienewsi</i>	–	250	250	208	ND
	<i>E. hellem</i>		279	133 + 146	200	99.6 ^e
9	<i>E. bienewsi</i>	–	250	250	208	99.6 ^d
	<i>E. hellem</i>		279	133 + 146	200	99.6 ^e

^a Length of DNA fragments amplified with primer pair VI/PMP2.

^b Homology with the indicated DNA sequence from GenBank.

^c ND, not done.

^d The *E. bienewsi* SSU rRNA sequence (accession no. AF024657) was used for comparison.

^e The *E. hellem* SSU rRNA sequence (accession no. AF039229) was used for comparison.

and without diarrhea by light microscopy and identified only one case of intestinal microsporidiosis due to *E. bienewsi* (21). Another study examined 40 European travelers from the tropics with a clinical picture of protracted diarrhea and identified four cases of imported *E. bienewsi* infection: one HIV-infected short-term traveler, a pregnant long-term traveler, and two immunocompetent short-term travelers. Diarrhea was self-limited, and the spores cleared from the stools in all non-HIV-infected travelers but showed a chronic course in the HIV-infected one. Infections were diagnosed by light microscopy with confirmation by PCR (13). Spores of microsporidia were detected by light microscopy in the stools of four French travelers presenting clinically with chronic diarrhea. Molecular identification of microsporidian species was based on the PCR amplification of an SSU rRNA sequence followed by *Hinf*I endonuclease restriction. *E. intestinalis* microsporidiosis was thus shown in two of the four patients examined (17). These three studies employed only light microscopy for detection of microsporidia in stools, and molecular-based techniques, such as PCR, were used only for confirmation and/or species differentiation. None of these studies used PCR for the examination of all stool samples. In our study, as well as in a previous study of members of our group (16), some of the cases were diagnosed only by PCR amplification. In a study that evaluated the prevalence of intestinal parasites in patients with diarrhea and AIDS in Zimbabwe, microsporidia were found by light microscopy in 10 out of 55 (18%), whereas PCR detected microsporidia in 28 out of 55 patients (51%) (10). Probably the limited sensitivity of light microscopy is responsible for these results. The detection limit of light microscopy has been determined to be between 10⁴ and 10⁶ microsporidian spores per g of stool, whereas PCR is able to detect spore concentrations as low as 10² per g of stool (16, 18). Thus the true prevalence of microsporidia has to be determined by highly sensitive techniques, such as PCR. However, diagnostic approaches regarding diarrhea are usually done late in the course of the disease, at a time when spore excretion in the stool may have decreased. In all of our cases except one, only low numbers of spores were seen by

light microscopy. Interestingly, the two patients in our study with confirmed double infection due to *E. bienewsi* and *E. hellem* were a traveler and his daughter who returned from Singapore. In stool samples of the mother who accompanied the two patients to Singapore, no microsporidia were detected by light microscopy or by PCR. To the best of our knowledge this is the first report of *E. hellem* in stool samples and the first report of *E. hellem* in non-HIV-infected patients. Our study shows that intestinal microsporidiosis seems to be an underappreciated cause of travelers' diarrhea. New interest in these organisms by clinicians caring for travelers returning from tropical areas and improved diagnostic approaches should now help to increase our knowledge regarding the role of microsporidia in travelers' diarrhea.

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