

Occurrence of intestinal microsporidia in immunodeficient patients in Poland

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Abstract

Microsporidial infections may be asymptomatic in immunocompetent hosts, but can be severe and disseminated in HIV/AIDS patients, children, the elderly, or in immunocompromised individuals, including those with primary or medically-induced immunodeficiencies. 209 faecal samples were collected from 80 clinical patients, with or without abdominal symptoms, and tested for the presence of the parasites. Microsporidia were found in 10 of the 80 patients (12.5%) using trichrom staining of faecal smears and/or PCR. *Encephalitozoon intestinalis* and 1 unidentified species were identified in 2 of the 32 children with primary immunodeficiencies (6%), presenting with diarrhoea, including one co-infection with *Cryptosporidium meleagridis*. In the group of patients with medically-induced immunosuppression (transplant recipients), 8 of the 48 patients (17%) were tested positive for microsporidia. Thus, these pathogens should be taken into account when the other etiological agents cannot be found in diarrheic patients with PIDs or undergoing immunosuppressive treatment before or after transplantation. This article presents the results of the first epidemiological study on the occurrence and prevalence of microsporidia in patients with primary and secondary immunodeficiency in Poland.

Key words

Enterocytozoon bieneusi, *Encephalitozoon*, microsporidia, primary immunodeficiency, transplant recipients, intestinal infections, Poland

INTRODUCTION

Microsporidia are a group of intracellular parasites which have attracted the interest of parasitologists for over 100 years. The first species, *Nosema bombycis*, was discovered in the middle of the 19th century as the cause of silkworm disease (i.e., pepper disease, pébrine disease), which nearly destroyed the silkworm industry in Southern Europe [1, 2]. Since then, more than 1,400 microsporidian species have been described in both invertebrate and vertebrate hosts. Only 7 genera and 15 species of microsporidia have been associated with human infections [3, 4, 5]. The first report on human microsporidia infection was in 1959 and described the case of a 9-year-old Japanese boy who presented with disseminated microsporidiosis associated with fever, headache, vomiting, and spastic convulsions [6]. Interest in this group of parasites started with the development of the AIDS pandemic around the world in 1980's. In 1985, a new species *Enterocytozoon bieneusi* was found in an HIV-infected patient [7]. Since then, species of microsporidia have been recognized worldwide as etiologic agents of opportunistic infections. The clinical course of microsporidiosis depends on the immune status of the patient and the site of infection. The groups at risk constitute people with HIV/AIDS, organ transplant recipients being treated with immunosuppressive drugs, travellers, children and the elderly [8, 9, 10, 11, 12, 13]. Microsporidiosis may present as persistent or chronic diarrhoea (*Enterocytozoon*

bieneusi, *Encephalitozoon intestinalis* infection), urinary, pulmonary or disseminate infections that include sinusitis, encephalitis, tracheobronchitis, interstitial nephritis, hepatitis or myositis (*E. intestinalis* and other *Encephalitozoon* species). Increasingly, ocular microsporidiosis and microsporidial stromal keratitis are identified, particularly in contact lens users [2, 14].

Two species, *E. bieneusi* and *E. intestinalis* are responsible for the majority of the gastrointestinal and biliary tract infections in humans. They may cause severe persistent diarrhoea with malabsorption and weight loss, but asymptomatic infections have also been reported in immunocompetent persons [15, 16]. Routine diagnosis is based on light microscopy observation of chromotrope-based stained faecal smears, or another optically-brightening agent such as Uvitex 2B or Fungifluor [17, 18]. However, the differentiation of the microsporidian species is not solely based on spore observation and measurements; thus, PCR-based methods are recommended for this task, due to their sensitivity and specificity. Serological assays are not useful in diagnosis, however, they may be used for epidemiologic surveys [18, 19, 20].

Human microsporidiosis occurs worldwide, but data on the prevalence and geographic distribution of microsporidian infections are still incomplete and very diverse due to the use of different diagnostic methods and host subgroups [1, 5, 21]. Although many papers report microsporidian infection in HIV/AIDS patients, the epidemiological studies in other risk groups are still scarce [14, 16, 21]. In Poland, detection of microsporidia is not routinely performed in clinical practice, and only single cases of human microsporidiosis have been

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reported to date by scientists [22]. To our knowledge, no epidemiological studies have been completed in the area of Poland to date.

This paper presents the results of a study on the occurrence of intestinal microsporidia in 2 groups of patients: children with primary immunodeficiencies (PIDs) and clinical patients with medically-induced immunosuppression.

MATERIALS AND METHOD

Stool specimens. During a 7-year period (2002–2008), 209 samples were collected from 80 patients in 3 hospitals in Poland: the Children's Memorial Health Institute (CMHI), Institute of Haematology and Transfusion Medicine, both in Warsaw, and the Institute of Transplantology at the Medical University of Warsaw. Patients were divided into 2 groups on the basis of their age and immunological status:

Group 1. consisted of 31 children and 1 young man, who were patients from the CMHI. They presented impaired immunity due to various primary immunodeficiencies (PIDs as CD40L deficiency= hyper IgM syndrome;

Table 1. Characteristics of intestinal microsporidial infections in immunodeficient and immunosuppressed patients

No. and origin of samples	Gen-der	Age (year)	Immuno-logical status	Type & status of trans-plant	Chr_2R results	PCR results	Diarrhea	Species
9/02	M	6	PID, (Hyper IgM)	bone marrow	ND	+	yes	<i>Entero-cytozoon/Encephalitozoon</i>
36/07	M	29	PID, (Hyper IgM)	nd	ND	+	no	<i>E. intestinalis</i>
04/tra	F	50	IS	kidney / after	MS spores	neg	no	not determined
07/tra	M	54	IS	liver / after	MS spores	neg	no	not determined
51/hem	M	34	IS	bone marrow / before	MS spores	neg	no	not determined
52/hem	F	44	MD	bone marrow / before	MS spores	neg	no	not determined
53/hem	M	28	IS	bone marrow / before	MS spores	neg	no	not determined
54/hem	M	21	IS	bone marrow / before	MS spores	+	yes	<i>E. intestinalis</i>
55/hem	M	25	IS	bone marrow / after	neg	+	no	<i>E. intestinalis</i>
56/hem	M	39	IS	bone marrow / before	MS spores	neg	no	not determined

Chr_2R – trichrom stain/IS – immunosuppression

MD – moderate immunodeficiency

MS spores – presence of spores on slides

ND – not done

PID – primary immunodeficiency

No. 04/tra and 07/tra – samples from patients of the Institute of Transplantology, Medical University, Warsaw, Poland

No. 51–56 with part –hem' are samples from patients of the Institute of Haematology and Transfusion Medicine, Warsaw, Poland

SCID, Omenn syndrome; Nijmegen breakage syndrome), and/or immunosuppressive treatment before or after organ transplantation (bone marrow or liver). Patients in this group presented with diarrhea or showed features of sclerosing cholangitis and hepatitis (Tab. 1).

Group 2. consisted of 48 adult patients subjected to medical immunosuppression before or after organ transplantation (kidney, liver or bone marrow). These patients rarely manifested intestinal disorders (Tab. 1). Faecal samples were collected from all patients on 2, 3 or more occasions, and stored at +4°C. The samples had been previously checked for *Cryptosporidium* and *Giardia* (oo)cysts by means of an immunofluorescent assay (IFA) and nested-PCR [24, 25].

Staining of faecal smears. Faecal smears were made from fresh stool specimens, air dried, fixed in methanol and stained with modified Weber's chromotrope-based staining-trichrom staining [26] (Chromotrope 2R Para-Pak Trichrome Stain, Meridian Diagnostics, Cincinnati, OH, USA). Smears were examined under oil immersion (x1,000 magnification). Pinkish-red oval objects measuring <2 µm in length, with a characteristic posterior vacuole and belt-like stripe in the middle, were identified as microsporidian spores.

Molecular methods. For the DNA extraction, stool specimens were first concentrated by modified Sheather's sucrose flotation [27]. DNA extraction and purification were carried out using QIAamp DNA Stool Mini Kit (Qiagen). Three sets of primers were used for PCR amplification.

Initially, to screen for the range of human-infecting microsporidial species including *E. cuniculi*, *E. hellem*, *E. intestinalis* and *E. bienewisi*, the 'general' primers by Raynaud et. al. [28] were used to amplify a 1,200 bp conserved region of the small-subunit (SSU) rRNA gene. These were: forward primer C1modyf (5'-CACCAGGTTGATTCTGCCTG-3') and reverse primer C2modyf (5'-GACGGGCGGTGTGTACAAAG-3').

Species-specific primers SINTF (5'-TATGAGAAGTGAGTTTTTTTC-3') and SINTR (5'-CCGTCCTCGTTCTCCTGCCCG-3') were then used to amplify a region of 545 bp from the SSU-rDNA of *E. intestinalis* [29]. Species-specific primers EBIEF (5'-GAAACTTGTCCACTCCTTACG-3') and EBIER (5'-CCATGCACCCTCCTGCCATT-3') were used to amplify a 607 bp fragment of the SSU-rDNA of *E. bienewisi* [30].

PCR conditions for C1modyf / C2modyf primers were: an initial denaturation at 94°C for 5 min.; then 35 cycles as follows: denaturation at 94°C for 1 min, annealing at 56°C for 1 min and elongation at 72°C for 1 min; with 5 min of final elongation at 72°C.

For 2 sets of species-specific primers, the PCR conditions were: an initial denaturation at 94°C for 5 min.; then 35 cycles as follows: denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and elongation at 72°C for 90 sec, with 5 min of final elongation at 72°C.

PCR reactions were conducted in a volume of 20 µl reaction mixture, in 2 repetitions for 3 and 5 µm of target DNA. Reactions were performed in a final volume of 20 µl and contained 0.33 mM dNTPs (Eurobio, Lille, France), 2 mM MgCl₂, 1×PCR buffer, 1 U Taq polymerase (Fermentas), 1 µM of each primer and 3 or 5 µl of extracted DNA. Two sets of controls, positive and negative, were incorporated in each PCR turn. Amplicons were visualized with Midori

Green stain (Nippon Genetics Europe GmbH) following electrophoresis in 1.5% agarose gels.

RESULTS

Prevalence of microsporidial infections in patients. Ten microsporidial infections were detected in 80 patients (12.5%), including 6 positive only through to microscopical examination of faecal smears and/or 4 positive by PCR (Tab. 1).

In the first group of patients, 2 of the 32 individuals with PIDs (1 child and 1 young man) were identified as positive (6.3%) on the basis of positive results of PCR:

Patient No. 36/07 – infection with *Enterocytozoon/Encephalitozoon* spp. was identified using ‘general’ primers C1modyf/ C2 modyf, and the microsporidian species was determined as *E. intestinalis* using species-specific primers SINTF/SINTR.

Patient No. 9/02 – diagnosed as infected with *Enterocytozoon/Encephalitozoon* spp. using ‘general’ primers C1modyf/ C2 modyf. No positive results were obtained for *E. bieneusi*-specific EBIEF/EBIER primers.

In this group of patients, trichrom staining of faecal smears was only carried out for 10 patients hospitalized during 2007–2008. All samples examined by light microscopy were negative.

In the second group of patients, with medically-induced immunosuppression, all samples were examined by 2 methods, trichrom staining and PCR. Eight out of 48 (16.7%) patients were positive for microsporidia on the basis of trichrom staining (7 cases) and/or PCR (2 cases) (Tab. 1). In patient No. 54 – microsporidian infection / hem was confirmed on the basis of 2 methods, light microscopy and PCR (Tab. 1).

All smears positive by light microscopy were positively verified in the laboratory of the Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA.

Two patients were diagnosed as infected with *E. intestinalis* using the species-specific primers SINTF/SINTR (Tab. 1). No positive PCR results were obtained for ‘general’ C1modyf/ C2modyf or *E. bieneusi*-specific EBIEF/EBIER primers sets in this group of patients.

Co-infection with *E. intestinalis* and *Cryptosporidium meleagridis*. In the first group of patients with PIDs, *E. intestinalis* infection was detected in patient No. 9/02, who had CD40L deficiency (hyper IgM syndrome) and was previously documented as the first case of *C. meleagridis* infection in Poland [24, 25]. *Cryptosporidium meleagridis* infection in this patient lasted for up to 29 months, despite applied treatment [24]. Following his third and the first successful bone marrow transplantation (HSCT), the patient recovered and became free of both *Cryptosporidium* and microsporidian parasites.

Symptomatic and asymptomatic infections. Diarrhea and/or another abdominal symptoms were reported by 25 of the 80 patients involved in the study, including 21 patients with PIDs from the first group and 4 patients with medically-induced immunosuppression from the second group (before or after organ transplantation). Only 2 patients in whom *E. intestinalis* infection was identified, presented various

abdominal symptoms. The other microsporidian infections (n=8) detected in these patients remained asymptomatic.

DISCUSSION

The main aim of the presented paper was to carry out the first epidemiological study in Poland on the occurrence of microsporidia in 2 groups of patients at risk of microsporidiosis, with impaired immune systems (PIDs and transplant patients). By applying 2 methods for the detection of intestinal infections, the overall prevalence of microsporidia, mainly *E. intestinalis*, was determined as 12.5%. Prevalence was higher in the group of transplant recipients in comparison to children with PIDs (17% and 6%, respectively). These values are very similar to the prevalence found in patients with chronic diarrhea, which was in the range of 6 – 17% for children and elderly people [12, 31, 32, 33, 34]. In other studies, when the seroprevalence was determined, the values ranged from 1.3% – 8% in immunocompetent persons, such as blood donors and animals breeders [20]. Prevalence of microsporidian infections in HIV/AIDS patients may be higher and varies from approx. 5% – 50%, with overall prevalence of about 15% [4, 10, 35, 36]. In Poland, detection of microsporidia is not routinely carried out in clinical diagnostics, and only single cases of infection in humans have been reported to date [22, Bednarska – GenBank, Accession No. JN107808].

Recently, *E. intestinalis* infections were identified in 2 lemur species from the Zoological Garden in Poznań, Poland, confirming the existence of a zoonotic reservoir of the pathogens [37]. This study presents the first epidemiological data on these important opportunistic pathogens in immunocompromised, non-HIV/AIDS patients. To the best of our knowledge, this is also the first report on microsporidia infections in patients with PIDs, confirmed by PCR technique. In an earlier study, Aoun et al. [38] identified microsporidia in 2 boys with PIDs (including 1 with hyper IgM syndrome, as in the presented study) by microscopical observation of Chromotrop-stained faecal smears.

Among non-HIV/AIDS patients and immunosuppressed individuals, such as organ transplant recipients, children, and patients with chronic diseases, microsporidiosis is manifested rather as self-limited diarrhea or can be asymptomatic [16, 21, 39]. In the presented study, only 2 patients manifested intestinal disorders, including 1 child with CD40L deficiency (hyper IgM syndrome) and long-lasting *C. meleagridis* infection, that may have been responsible for the diarrhea [24, 25]. The second symptomatic patient was characterized by a relatively high intensity of infection, confirmed by positive results from 2 detection methods. Interestingly, recently, a study showing predominance of asymptomatic infections with different species/strains of *Encephalitozoon* and *E. bieneusi* was published on the basis of epidemiological studies in a healthy Czech population [16].

In the presented study, species-specific PCR revealed only 1 microsporidian species in 3 patients positive by PCR–*E. intestinalis*. However, the species of microsporidia involved in the 7 cases identified only on the basis of microscopical observation of faecal smears or ‘general’ primers could not be determined; therefore, infections with *E. bieneusi* cannot be excluded. In developing countries, *E. bieneusi* prevalence rates for HIV/AIDS patients with diarrhea are between 2.5%

– 51% [16], and < 5% for patients without diarrhea have been reported [40]. For comparison, *Encephalitozoon* sp. infections are usually rarer, reaching a maximum of 10% [41]. In Poland, only recently, the first case of *E. bienewsi* infection in a transplant patient was identified [22]; therefore the higher occurrence of *Encephalitozoon* is not surprising.

Microsporidia were more common in the current study in the group of transplant recipients. There are less than 100 cases of microsporidiosis reported in these types of patients worldwide, and microsporidian species were identified only in about 55% of these cases [9, 42, 43, 44]; the majority of the infections were diagnosed as *E. bienewsi* infections, and associated with intestinal disorders. *Encephalitozoon* spp. were rarely detected (about 10%) and the majority of these cases were found in kidney transplant recipients [9]. In these patients, disseminated but asymptomatic (without diarrhea) infections were common. Interestingly, in the majority of asymptomatic microsporidian infections, the species of parasite was not determined.

Presented above are the results of the first epidemiological study in Poland on microsporidia prevalence in patients with primary or secondary immunodeficiencies. The occurrence of microsporidia at the level of 12% suggests that these pathogens should be taken into account when other etiological agents cannot be found in diarrheic patients with PIDs, or those undergoing immunosuppressive treatment before or after transplantation. Additionally, asymptomatic but quite common infections of microsporidia may constitute a health risk factor in cases of advanced immune system impairment, i.e. due to co-infection in PIDs or medically-induced.

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