Abstract

**Background:** Microsporidia (Fungi) have been repeatedly identified as the cause of opportunistic infections predominantly in immunodeficient individuals such as AIDS patients. However, the global epidemiology of human microsporidiosis is poorly understood and the ability of microsporidia to survive and multiply in immunocompetent hosts remains unsolved.

**Aims:** To determine the presence of latent microsporidial infections in apparently healthy humans in the Czech Republic, the authors tested sera, urine and stool originating from fifteen persons within a three month period examined on a weekly basis.

**Methods:** Sera, stool and urine samples originating from fifteen HIV-negative people at risk with occupational exposure to animals, aged 22–56 years, living in the Czech Republic were tested by indirect immunofluorescence assay (IFA) for the presence of specific anti-microsporidal antibodies, standard Calcofluor M2R staining for the detection of microsporidian spores in all urine sediments and stool smears and molecular methods for the microsporidial species determination.

**Results:** Specific anti-microsporidal antibodies were detected in fourteen individuals, asymptomatic *Encephalitozoon* spp. infection was found in thirteen and *E. bieneusi* infection was detected in seven of those examined. While *E. hellem* 1A and *E. cuniculi* II were the major causative agents identified, seven different genotypes of *E. bieneusi* were recorded.

**Conclusions:** These findings clearly show that exposure to microsporidia is common and chronic microsporidiosis is not linked to any clinical manifestation in healthy population. Moreover, our results indicate much higher incidence of microsporidial infections among an apparently healthy population than previously reported. These results open the question about the potential risk of reactivation of latent microsporidiosis in cases of immunosupression causing life-threatening disease.

---

**Introduction**

Microsporidia have emerged as causative agents of opportunistic infections in AIDS patients and other immunodeficient individuals. Several species of microsporidia can cause disease in humans. Intestinal microsporidiosis due to *Encephalitozoon* (*Septata*) intestinalis and *Enterocytozoon* bieneusi are most frequently reported among immunocompromised people including patients with acquired immune deficiency syndrome (AIDS) [1,2] and other immunocompromised patients such as transplant recipients [3–6]. *Encephalitozoon* cuniculi and *E. hellem* are less prevalent among immunodeficient patients [7,8]. Infections with microsporidia in immunocompetent individuals such as travelers have also been described [9,10].

Although the most common clinical symptoms related to *Encephalitozoon* among immunodeficient patients are chronic diarrhea and malabsorption, they can also cause systemic diseases. While immunocompetent persons often have mild or self-limiting disease, AIDS patients can experience weight loss and increased mortality [11].

Since the studies examining the prevalence of microsporidiosis have been limited to patients who are infected with human immunodeficiency virus (HIV) or who have AIDS, the global epidemiology of human microsporidiosis is poorly understood. Variation of spore shedding intensity of microsporidia was shown in both human and animals [12–16]. However, to our knowledge there have been no reports on the spore shedding pattern of microsporidia in immunocompetent humans. Therefore we aimed
Materials and Methods

Ethics statement

The study was approved by the Hospital České Budějovice ethics committee (protocol no. 202/07). Written informed consent was obtained from every person prior to examination.

Stool samples

Between September and December 2007, a total of 180 individual stool and 180 urine samples were collected on the weekly basis for 3 months from fifteen HIV-negative people at risk of occupational exposure to various animals, such as farm ruminants, pigs, poultry and rodents. The male to female ratio was 8 (53%) to 7 (47%) with mean age of 33 ± 11 years and range between 22–56 years. The samples were stored at 4°C in PBS and compared with negative and positive control sera. Sera with positive fluorescence at titers greater than 128 were considered positive.

Serological examination

Prior to the study, serum samples were obtained from all individuals included and the presence of specific anti-microsporidial immunoglobulin G was tested by indirect immunofluorescence assay (IFA). IFA was performed with purified whole spores of E. hellem, E. cuniculi or E. intestinalis grown in vitro in VERO E6 cells and semi-purified spores of E. bieneusi at the concentration 10⁶ spores/well (spores kindly provided by Dr. G.S. Visvesvara, CDC Atlanta, GA, USA). Sera were serially diluted (1:8, 1:16, 1:32, 1:64, 1:128 and 1:256) in PBS and compared with negative and positive control sera. Serum specific antibodies against microsporidial infection were detected by IFA. Sera from five individuals were positive for microsporidia spores, which were subsequently molecularly characterized as E. bieneusi.

Microscopic examination

Standard Calcofluor M2R staining [17] was used for the detection of microsporidian spores in all urine sediments and stool smears. Stained slides were examined by fluorescence microscopy using UV light with a wavelength of 490 nm and at a magnification of 1000×. Positive control slides were used for each examination.

Examination by molecular methods

The DNA was isolated from the stool and urine samples using homogenization by bead disruption using FastPrep–24 Instrument (MP Biomedicals, CA, USA) and DNA was extracted using commercially available isolation kit QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. Acquired DNA was stored at −20°C.

The nested PCR protocol by Katzwinel-Wladarsch et al. [18] amplifying the ITS region of E. bieneusi and E. cuniculi using microsporidia-specific primers was performed as described elsewhere [16]. As positive controls the following were used: DNA obtained from spores of E. intestinalis originally isolated from AIDs patients [19] and grown in vitro in VERO E6 cells in the Laboratory of Veterinary and Medical Protistology at the Institute of Parasitology ASCR, and DNA from spores of E. bieneusi of genotype D originally isolated from a pig [16]. PCR products were visualized on a 2% agarose gel containing 0.2 μg/ml ethidium bromide and directly sequenced on the ABI3730XL sequence analyzer (Applied Biosystems, Foster City, CA). Sequences were aligned and completed using programs ChromasPro (Technelysium, Pty. Ltd) BioEdit and Clustal X 2.0.6 and compared with sequences in GenBank.
Table 1. Seropositivity to *Enterocytozoon bieneusi* and *Encephalitozoon* spp. at the beginning of the tested period.

| Personal identification number/control sera | E. hellem | E. intestinalis | E. cuniculi | Enterocytozoon bieneusi |
|--------------------------------------------|-----------|----------------|------------|-------------------------|
| 1                                          | 1:64      | 1:64           | 1:128      | 1:64                    |
| 2                                          | 1:128     | 1:32           | 1:128      | 1:64                    |
| 3                                          | 1:64      | 1:64           | 1:128      | 1:64                    |
| 4                                          | 1:64      | 1:32           | 1:128      | 1:64                    |
| 5                                          | 1:128     | 1:16           | 1:128      | 1:64                    |
| 6                                          | 1:32      | 1:16           | 1:128      | 1:32                    |
| 7                                          | 1:128     | 1:32           | 1:128      | 1:32                    |
| 8                                          | 1:128     | 1:64           | 1:128      | 1:32                    |
| 9                                          | 1:128     | 1:64           | 1:128      | 1:64                    |
| 10                                         | 1:32      | 1:16           | 1:32       | 1:128                   |
| 11                                         | 1:32      | 1:16           | 1:32       | 1:128                   |
| 12                                         | 1:128     | 1:16           | 1:128      | 1:32                    |
| 13                                         | 1:32      | 1:32           | 1:32       | 1:32                    |
| 14                                         | 1:64      | 1:16           | 1:64       | 1:32                    |
| 15                                         | 1:128     | 1:32           | 1:128      | 1:64                    |
| Negative serum 1                           | 1:8       | 1:16           | 1:32       | 1:8                     |
| Negative serum 2                           | 1:16      | 1:16           | 1:32       | 1:32                    |
| Negative serum 3                           | 1:8       | 1:32           | 1:32       | 1:16                    |
| Positive serum 1                           | 1:256     | 1:32           | 1:16       | 1:32                    |
| Positive serum 2                           | 1:32      | 1:128          | 1:64       | 1:32                    |
| Positive serum 3                           | 1:32      | 1:128          | 1:256      | 1:32                    |
| Positive serum 4                           | 1:8       | 1:32           | 1:32       | 1:128                   |

Figure 1. Frequency of microsporidia excretion by naturally infected immunocompetent humans. A urine sediments; B stool samples. doi:10.1371/journal.pntd.0001162.g001
because they are not specifically searched for in most diagnostic labs, they are rather small, and their staining with hematoxylin and eosin is not sufficient. Most of what is now known about human microsporidiosis can be attributed to the experience with patients infected with HIV [1,2]. However, with increased awareness and improved diagnostics, microsporidia have become more frequently reported also in immunocompetent individuals, producing asymptomatic infections [20–22]. Despite limited sample number our findings showed a well-supported correlation between spore presence in excretions and seropositivity, which discriminates the actual latent microsporidiosis from simple consumption and passage of spores through the intestinal tract.

Intermittent spore shedding for a long period has been experimentally demonstrated for several hosts including rabbits with E. cuniculi [12], wild-type mice with E. intestinalis [13], pigs with E. bieneusi [15], budgerigars with naturally acquired Encephalitozoon spp. infection [23] and HIV-positive patients with E. bieneusi [14]. The persistence of microsporidia despite resolution of the intestinal disorder suggests that microsporidia infection may cause clinical symptoms (e.g., diarrhea) during the early stages of infection that could be overlooked and resolved even though the microsporidia persist.

Our survey was performed on a limited sample size from a highly selected population, which could result in decreased statistical power. On the basis of present results it is obvious, that prevalence data of microsporidial infection reported by various authors reaching up to 38% the case of Encephalitozoon spp. and 51% for E. bieneusi, could be hampered by collection of only a single sample for diagnosis, especially in low level infections. While the twelve week sampling enabled us to detect E. cuniculi in 86% of tested people, E. hellem in 66% and E. bieneusi in 47%, the hypothetical individual single sampling performed at any day would identify E. cuniculi in only 0–27% of persons, E. hellem in 0–13%, and E. bieneusi in 0–13%.

Based on data in the literature and our experience, it seems that the incidence of microsporidial infections is much higher than previously reported and microsporidia may represent neglected etiological agent of more common diseases. However, it is not known how extensive such silent infections are in asymptomatic carriers, including both humans and animals, which have been reported increasingly to harbour various species and genotypes of microsporidia [16,24,25]. Moreover, the fact that microsporidia DNA were detected in urine sediments suggests, that microsporidia are able to disseminate also in immunocompetent hosts despite previously reported protective T-cell mediated adaptive immunity together with several components of innate immunity [26,27]. Furthermore, the majority of prevalence studies currently rely on detection of spores in stool samples only. The results of this study clearly showed that infected seropositive person could excrete detectable amount of microsporidial DNA via urine, nevertheless examination of stool sample will be negative. Detection of specific antibodies seems to be more sensitive than one-shot detection of spores and can provide more accurate information about ongoing microsporidia infection.

In conclusion, studies focusing on the epidemiology of microsporidiosis will more clearly define the environmental sources of microsporidia that pose a risk for transmission so that preventative strategies can be implemented. Since no data exist about latent infection in immunocompetent carriers, possible infection reactivation in these individuals and person to person transmission risk via organ donation, such epidemiological data must be compared with experiments that could solve this question definitively. Moreover, using detection methods with a high sensitivity, such as PCR, and consecutive sampling from every individual is recommended to provide more precise epidemiological data.

Supporting Information

Checklist S1 STROBE checklist. (DOC)

Acknowledgments

This work could not be done without the willingness and close collaboration with tested people. We are grateful to all of them for providing us with samples. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the CDC.

Author Contributions

Conceived and designed the experiments: BS MK. Performed the experiments: BS DK KS. Analyzed the data: BS ZK. Contributed reagents/materials/analysis tools: DK ZK KS. Wrote the paper: BS MK.

Table 2. Detection of microsporidia in urine and stool samples of naturally infected immunocompetent humans.

| Species | genotype | Number of positive person | Number of positive specimens | Reference sequence |
|---------|----------|---------------------------|-----------------------------|-------------------|
| E. hellem | 1A | 10 | 10 | 9 | AF338367 |
| E. cuniculi | I | 1 | 1 | 0 | AF338410 |
| E. bieneusi | BRmv2 | 1 | 1 | 1 | EU849132 |
| | H | 1 | 1 | 1 | AF135835 |
| | EpbC | 1 | 0 | 1 | AF076042 |
| | H/F | 1 | 1 | 1 | EU849130 |
| | CZ4 | 1 | 2 | 0 | HM143725 |
| | CZ5 | 1 | 0 | 1 | HM143726 |
| | CZ6 | 1 | 0 | 1 | HM143727 |

doi:10.1371/journal.pntd.0001162.t002
References

1. Dowd SE, Gerba CP, Enriquez FJ, Pepper IL (1998) PCR amplification and species determination of microsporidia in formalin-fixed feces after immunomagnetic separation. Appl Environ Microbiol 64: 333–336.
2. Kotler DP, Orenstein JM (1998) Clinical syndromes associated with microsporidiosis. Adv Parasitol 40: 321–349.
3. Orenstein JM, Chiang J, Steinberg W, Smith PD, Rotterdam H, et al. (1990) Intestinal microsporidiosis as a cause of diarrhea in human immunodeficiency virus-infected patients: a report of 20 cases. Hum Pathol 21: 475–481.
4. Sax PE, Rich JD, Picciati WS, Trnka YM (1995) Intestinal microsporidiosis as a cause of diarrhea in human immunodeficiency syndrome. Arch Pathol Lab Med 116: 660–668.
5. Mohindra AR, Lee MW, Visvesvara GS, Moura H, Parasuraman R, et al. (1995) Identification of microsporidia in patients. Folia Parasitol 45: 108–112.
6. Gamboa-Dominguez A, De Anda J, Donis J, Ruiz-Maza F, Visvesvara GS, et al. (2002) Disseminated microsporidiosis in a renal transplant recipient. Transplant Infect Dis 4: 102–107.
7. Kucerova-Pospisilova Z, Ditrich O (1998) The serological surveillance of several groups of patients using antigens of Encephalitozoon helmot and E. canis vaccines in patients. Folia Parasitol 45: 108–112.
8. Schwartz DA, Bryan RT, Hewan-Lowe KO, Visvesvara GS, Weber R, et al. (1992) Disseminated microsporidiosis (Encephalitozoon helmot) and acquired immunodeficiency syndrome. Arch Pathol Lab Med 116: 660–668.
9. Raynaud L, Delbac F, Broussolle V, Rabodonirina M, Girault V, et al. (1998) Detection of microsporidia in travelers with diarrhea. J Clin Microbiol 36: 37–40.
10. Muller A, Bialek R, Kamper A, Falkenhauer G, Salisberger B, et al. (2001) Detection of microsporidia in travelers with diarrhea. J Clin Microbiol 39: 1630–1632.
11. Waywa D, Kongkriengdaj S, Chaidatch S, Tiengrim S, Kowadisaiburana B, et al. (2004) Protozoan enteric infection in AIDS-related diarrhea in Thailand. Southeast Asian J Trop Med Public Health 35: 251–255.
12. Cox JC, Hamilton RC, Attwood HD (1979) An investigation of the route and progression of Encephalitozoon canis infection in a Mexican kidney transplant recipient. Transplantation 75: 1898–1900.
13. Achbarou A, Ombrouch C, Gueragenta T, Charlotte F, Remia L, et al. (1996) Experimental model for human intestinal microsporidiosis in interferon gamma receptor knockout mice infected by Encephalitozoon intestinalis. Parasite Immunol 18: 387–392.
14. Clarridge JE 3rd, Karlhanss S, Rabeneck L, Marino B, Foote LW (1996) Quantitative light microscopic detection of Encephalitozoon bieneusi in stool specimens: a longitudinal study of human immunodeficiency virus-infected microsporidiosis patients. J Clin Microbiol 34: 520–523.
15. Breitenmoser A, Mathis A, Buri G, Weber R, Deplazes P (1999) High prevalence of Encephalitozoon bieneusi in swine with four genotypes that differ from those identified in humans. Parasitology 118: 447–453.
16. Sak B, Kva M, Hanifiková D, Cama V (2003) First report of Encephalitozoon bieneusi infection on a pig farm in the Czech Republic. Vet Parasitol 153: 220–224.
17. Vávra J, Chalupský J (1982) Fluorescence staining of microsporidian spores with the brightener “Calcofluor White M2R”. J Protozool 29: 303.
18. Kutzwinkel-Wlaarsch S, Lieb M, Huse W, Loscher T, Rinder H (1996) Direct amplification and species determination of microsporidian DNA from stool specimens. Trop Med Int Health 1: 373–378.
19. Didier ES, Rogers LB, Orenstein JM, Baker MD, Vossbrinck CR, et al. (1996) Characterization of Encephalitozoon (Septata intestinalis isolates cultured from nasal mucosa and bronchoalveolar lavage fluids of two AIDS patients. J Eukaryot Microbiol 43: 34–43.
20. Tumwine JK, Kankimwa A, Nabukera N, Akiyoshi DE, Buckholz MA, et al. (2002) Encephalitozoon bieneusi among children with diarrhea attending Mulago Hospital in Uganda. Am J Trop Med Hyg 67: 299–303.
21. Nkinin SW, Asonganyi T, Didier ES, Kaneshiro ES (2007) Microsporidiosis infection is prevalent in healthy people in Cameroon. J Clin Microbiol 45: 2041–2046.
22. Samie A, Obi CL, Tzipori S, Weiss LM, Guerrant RL (2007) Microsporidiosis in South Africa: PCR detection in stool samples of HIV-positive and HIV-negative individuals and school children in Vhembe district, Limpopo Province. Trans R Soc Trop Med Hyg 101: 547–554.
23. Sak B, Kva M, Kvetonoha D, Ditrich O (2010) Microsporidia in exotic birds: intermittent spore excretion of E.bieneusi spp. in naturally infected budgerigars (Melopsittacus undulatus). Vet Parasitol 168: 196–200.
24. Dengel BM, Zahler M, Hermans W, Heinritz K, Spillmann T, et al. (2001) Zoonotic potential of Encephalitozoon bieneusi. J Clin Microbiol 39: 4495–4499.
25. Buckholz MA, Lee JH, Tzipori S (2002) Prevalence of Encephalitozoon bieneusi in swine: an 18-month survey at a slaughterhouse in Massachusetts. Appl Environ Microbiol 68: 2593–2599.
26. Moretto MM, Lawlor EM, Khan IA (2008) Aging mice exhibit a functional defect in mucosal dendritic cell response against an intracellular pathogen. J Immunol 181: 7977–7984.
27. Leitch GJ, Celallos C (2009) A role for antimicrobial peptides in intestinal microsporidiosis. Parasitology 136: 175–181.