

Occurrence of microsporidia as emerging pathogens in Slovak Roma children and their impact on public health

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Abstract

Introduction and objective: Microsporidia are identified as ubiquitous organisms of almost every animal group and are now recognized as emerging opportunistic pathogens of human. The risk factors include immunodeficiency, lack of sanitation, and exposure to contaminated water and infected animals. In Slovakia, the places with an increased risk of infection due to the presence of risk factors and routes of transmission are represented by Roma settlements. Therefore, the aim of this work was to study the occurrence of *Encephalitozoon* spp. and *E. bienersi* in children living in Roma settlements.

Materials and methods: Stool samples were examined of 72 clinically healthy children coming from a group of the non-integrated Roma minority for the presence of microsporidia *Encephalitozoon* spp. and *E. bienersi*. Microsporidian spores were detected by standard Rylux D, staining and by PCR and DNA sequencing.

Results: Of the total number of 72 stool smears examined, 22 were positive, which represented 30.6%. By the Real Time PCR, *E. bienersi* was detected in 3 samples (4.2 %) and *E. cuniculi* in 19 samples (26.4 %). By comparing the sequences with sequences in the GenBank, *E. cuniculi* genotype I (Accession No. AJ005581.1) and *E. bienersi* genotype A (Accession No. AF101197.1).

Conclusions: Microsporidia, as newly emerging pathogens of humans and animals, are characterised by the production of spores which are environmentally resistant. Diseases caused by them have a cosmopolitan occurrence. Although *E. bienersi* and *E. cuniculi* belong to the most frequently diagnosed species of microsporidia in humans, in Slovakia, this is the first confirmed evidence of *E. bienersi* genotype A, as well as *E. cuniculi* genotype I in humans by the molecular method.

Key words

microsporidia, *Enterocytozoon bienersi*, *Encephalitozoon cuniculi*, roma children, zoonotic potential

INTRODUCTION

Microsporidia are single-celled, obligate spore-forming intracellular pathogens that infect a broad range of invertebrates and vertebrates, including humans. Based on observations that include the presence of chitin in the spore wall, identification of a mitochondrial HSP70 gene, and phylogenetic analyses of genes encoding beta tubulin, large subunit RNA polymerase II, and TATA box binding protein, microsporidia were reclassified with the fungi [1].

Although the first human case of microsporidian infection was reported in 1959 [2], until the beginning of the AIDS pandemic in the 1980s, microsporidia were identified only rarely [3].

With the increasing number of HIV positive patients and patients with AIDS, as well as improvement of diagnostic methods, the prevalence of microsporidiosis also started to increase

To date, of the nearly 1,200 species of microsporidia belonging to the 150 genera that have been identified, 14 are known to infect humans [4]. Of these, the *Enterocytozoon bienersi*, *Encephalitozoon intestinalis* and *Encephalitozoon cuniculi* are the most frequently occurring.

Currently, microsporidia are considered as the cause of emerging and opportunistic infections, not only in persons with HIV/AIDS, but also in organ transplant recipients, children, travellers, the elderly, contact lens wearers, patients with malignant diseases and diabetics [5].

The risk factors associated with infection are not known precisely, but it is known for sure that they are very closely related to the immune status. The main risk factor of human microsporidiosis is immune deficiency [4]. Clinical signs of infection occur in people with CD4 T-lymphocyte count below 100 cells per mm³ blood, and include (depending on the species of microsporidia) intestinal, lung, kidney, liver, sinus, muscle, or eye disease, and occasionally also infection of CNS.

Other risk factors include eating undercooked beef at least once a month, occupational and recreational contact with contaminated water, animal exposure through direct contact, lack of sanitation, undernourishment, and homosexual practices [4].

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Transmission of microsporidia occurs primarily through faecal-oral routes with sources of infection including infected humans and animals. Other ways of transmission include inhalation of contaminated aerosols, direct contact with the eyes, broken skin, sexual transmission, and ingestion of contaminated food and water [4].

In Slovakia, Roma settlements are the places with an increased risk of infection due to the presence of risk factors and routes of transmission.

According to the Office of Government Plenipotentiary for the Roma Issue in Slovakia there are over 1,000 Roma settlements at present. Around 150,000 Roma (almost half under the age of 18) live in segregated places which are unfit for a proper lifestyle and have a negative impact on the health of the population. One of the critical areas is drinking water. In numerous settlements there is just a single well serving all the residents. The quality of water is not monitored regularly and people using the water often learn about its possible contamination only after the spread of infection [6].

Therefore, the aim of the presented study was to investigate the occurrence of *Encephalitozoon* spp. and *E. bienewisi* in the children living in Roma settlements in Eastern Slovakia.

MATERIALS AND METHODS

Study population. A total of 72 clinically healthy Roma children were examined for the presence of microsporidia *Encephalitozoon* spp. and *E. bienewisi*. Children coming from Roma settlements of Eastern Slovakia were divided into 4 groups according to their age. In the 1st group were children under the age one year (n=27), in the 2nd group were children aged between 1 – 5 years (n=19), in the 3rd group were children age between 6 – 9 years (n=17), and in the 4th group, children aged between 10 – 14 years (n=9) (Tab. 1).

Samples. For microscopic and molecular diagnostic stool samples, 2 from each child, were used. Samples for microscopic examination were stored at 4°C, and those for molecular analysis were frozen and stored at -18°C until processed.

Confocal fluorescence microscopy. A total of 72 stool samples from Roma children were screened for the presence of microsporidian spores by confocal fluorescence microscopy after Rylux D (Ostacolor, Czech Republic), stained as previously described [7].

Molecular analysis. DNA was extracted from unpreserved stool samples by following mechanical disruption. 200 µL of spores (100 µL spore suspension with 100 µL lysis buffer – NET 50 + sarcosine) was boiled for 30 min, immersed in liquid nitrogen and de-frosted at +100°C in turns. Extraction and purification of DNA by commercial isolation kit DNA Sorb B (Federal Budget Institution of Science, Moscow, Russia) were performed according to the manufacturer's instructions.

The *Encephalitozoon*-specific primer pair *ecfITSf/ecfITSr* for real-time SYBR Green amplification has been described by Malčeková et al. [8] and chosen to amplify the ITS region, part of the small subunit (SSU) rRNA and part of the large subunit (LSU) rRNA genes of *E. cuniculi*, *E. intestinalis* and *E. hellem* at an annealing temperature of 60°C. The species-specific primer pair *MSP3/MSP4B* was used for amplification

of the ITS region, part of the small subunit (SSU) rRNA, and part of the large subunit (LSU) rRNA genes of *E. bienewisi* at an annealing temperature of 54°C [9].

Amplifications were performed in 25 µL reactions containing 12.5 µL Fast Universal SYBR Green Master (Roche), 0.5 µL of each primer (30 pmol/µL) and 8 µL of template. PCR reactions were run in a BIOER Line-Gene thermocycler using a step cycle programme. After the initial step at 50°C for 2 min following initial denaturation of the DNA at 95°C for 10 min, 40 amplification cycles were run: denaturation 95°C for 15 sec and hybridisation 54 / 60°C for 1 min. A sample was considered to be positive if the melting temperature was the same or ± 0.5°C in comparison to a positive control.

PCR products were directly sequenced in both directions. Sequences were aligned and completed using Chromas Pro Programme, Bioedit and Custal X, and compared to known sequences in the National Centre for Biotechnology Information GenBank database.

Statistical methods. Basic descriptive statistics were used for the analysis of the obtained results. Relative risks (RR) and their 95 percent confidence intervals (95% CI) were estimated for the occurrence of microsporidia (*E. cuniculi* and *E. bienewisi*). All tests were considered significant at $p < 0.05$.

RESULTS

Of the total number of 72 stool smears examined, 22 were positive, which represented 30.6%. In all positive cases the spores of microsporidia were shown as oval, brightly fluorescent formations of approximately 1.5 µm × 3 µm (Fig. 1).

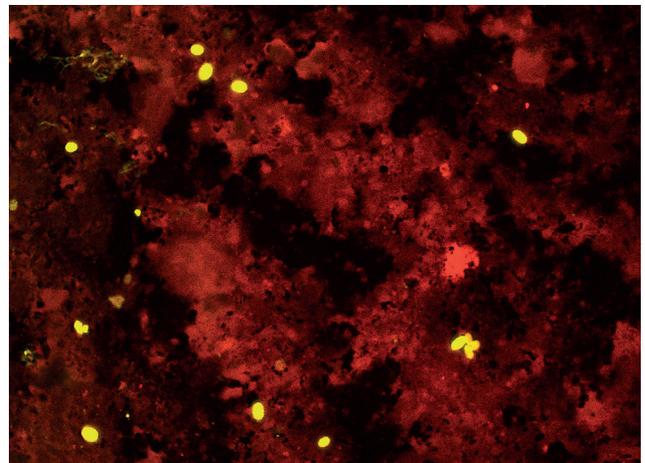


Figure 1. Spores *Encephalitozoon* spp. in faeces of the examined Roma children, visualised with optical brightener Rylux D (x 600).

Microsporidia was molecularly detected in 22 stool samples. The real-time SYBR Green PCR using *Encephalitozoon*-specific (*ecfITSf/ecfITSr*) and species-specific (*MSP3/MSP4B*) primer pairs revealed the presence of microsporidian species *E. cuniculi* in 19 samples (26.4%), and *E. bienewisi* in 3 samples (4.2%). No *E. intestinalis* and *E. hellem* positive samples were identified. Co-infections *E. bienewisi* and *E. cuniculi* were detected only in one child in the 6 – 9-old-age group (1.4%).

Table 1. Significant difference between positive cases of microsporidia in relation to gender and age.

Age	Boys		Girls		Σ		Relative risk (95% CI)	P-value
	N	P (%)	N	P (%)	N	P (%)		
< 1	19	6 (31.6)	8	1 (12.5)	27	7 (25.9)	2.5 (0.3 – 17.7)	0.4
1 – 5	12	5 (16.7)	7	1 (14.3)	19	6 (31.6)	2.9 (0.4 – 20.2)	0.3
6 – 9	11	5 (45.5)	6	1 (16.7)	17	6 (35.3)	2.7 (0.4 – 18.3)	0.3
10 – 14	5	1 (20.0)	4	2 (50.0)	9	3 (33.3)	0.4 (0.1 – 2.9)	0.4
Σ	47	17 (36.2)	25	5 (20.0)	72	22 (30.6)	1.8 (0.8 – 4.3)	0.2

Table 2. Significant difference between positive cases of *E. cuniculi* and *E. bienewsi* in relation to gender.

	Boys P (%)	Girls P (%)	Relative risk (95% CI)
<i>E. cuniculi</i>	14 (29.8)	5 (20.0)	1.68 (0.7 – 4.1)
<i>E. bienewsi</i>	3 (4.2)	0	4.3 (0.2 – 79.4)

Positive cases were observed in all of the examined groups, with the highest positivity in the group of 6 – 9-year-old children (35.3%). According to statistical evaluation, no significant differences between positive cases in individual age categories were observed (Tab. 1).

Upon comparing the relative risk of occurrence of microsporidian infection between boys and girls in individual age categories, it was found that the risk of infection to be 1.8-times higher in the group of boys than in the group of girls. The risk was 2.5-times higher for boys younger than one year, 2.9-times higher for boys between 1 – 5 years, and 2.7-times higher for 6 – 9 years old boys, compared with the girls. Only in the group of boys aged between 10 – 14 years existed a relative risk 0.4 times higher, compared with girls (Tab. 1).

Upon comparing the relative risk of positive cases of *E. cuniculi* and *E. bienewsi* in relation to gender, it was found that the risk of *E. cuniculi* infection to be nearly 1.7 times higher in the group of boys than in girls, and 4.3-times higher for boys compared with girls in the case of *E. bienewsi* infection (Tab. 2).

Positive PCR products were sent for sequencing. The sequences were compared with sequences in GenBank database. They were identical with the sequence of *E. cuniculi* genotype I (Accession No. AJ005581.1) and *E. bienewsi* genotype A (Accession No. AF101197.1).

DISCUSSION

Microsporidia as newly emerging pathogens of humans and animals are characterised by the production of spores which are environmentally resistant. Diseases caused by them have a cosmopolitan occurrence. Data on prevalence have varied greatly depending on the geographical region, the population studied and the diagnostic methods used [4].

In Slovakia, microsporidiosis was first diagnosed in farm rabbits [10]. Later, this disease was reported in dogs, mice, cows, goats, cats, sheep, and also in humans [11, 12, 13, 14, 15].

In the presented study, 72 stool samples from Roma children were examined for the presence of microsporidia. By the RT PCR, in 3 samples *E. Bienewsi* was detected, and in 19 samples *E. cuniculi*.

E. bienewsi was the first genus of microsporidia detected in humans. Since then, several hundred *E. bienewsi* intestinal infections have been documented in all parts of the world, predominantly in immunodeficient and immunocompromised patients, but also in immunocompetent persons. This microsporidia had previously been considered to be a pathogen found only in humans, but at present, its zoonotic potential is a possibility. The *E. bienewsi* genotype A (Accession No. AF101197.1) identified in the presented study is identical to the genotype detected for the first time in patient with AIDS in Germany [16]. Further evidence of its presence have appeared several times subsequently in Peru [17], the Czech Republic [18] and the Netherlands [19]. Until 2009, the host range was limited only to humans, but Kašičková et al. [18] proved the first presence of this species in animals (birds) in the Czech Republic. Although *E. bienewsi* is one of the most frequently diagnosed species of microsporidia in humans, in Slovakia it is the first evidence of *E. bienewsi* genotype A in humans by the molecular method.

E. cuniculi is also one of the most frequent microsporidia which infects a wide range of mammalian hosts, including humans [4]. In 1995, 3 strains of *E. cuniculi* were identified, but until 2011, only 2 have been described as human isolates. Strain I was originally isolated from rabbits and also described in humans [20], and strain III, originally isolated from dogs, was also identified in AIDS patients [21]. Strain II, originally isolated from mice and blue foxes, was detected first time in humans by Sak et al. [22] in 2011. Our examination confirmed the presence of *E. cuniculi* strain I (Accession No. AJ005581.1) in 19 of the 72 examined children. As in the case of *E. Bienewsi*, this is the first confirmed evidence of the occurrence of *E. cuniculi* in Slovakia in humans by the molecular method.

As mentioned previously, the specific population groups at risk for microsporidia include mainly people with disrupted immune status; among them HIV positive patients and children were reported as the groups mostly affected by the disease [23]. Generally, the prevalence of microsporidiosis in children ranged from 17.4% – 76.9% in HIV positive patients, and from 0.8 – 22.5% in the immunocompetent or apparently immunocompetent kids [24, 25]. One of the risk factors is poor sanitary conditions and inadequate personal, household, and community hygiene, which are responsible for the higher sensibility to infections, including microsporidiosis. In Slovakia, the occurrence of these conditions is typical for children coming from the non-integrated Roma minority. According to several studies in many settlements, which are often built on loose soils, there is a lack of sanitation in their drinking water, sewage, waste pits and landfills, and lack of garbage disposal. This are also preconditions for uncontrollable reproduction in animals, especially rodents, dogs and birds, which can be important sources of infection for humans. Because microsporidian spores are released into the environment via stools, urine and respiratory secretions, the possible sources of infection may be infected persons or animals. In the microsporidian species detected in the presented study – *E. cuniculi* and *E. Bienewsi* – both have been clearly documented as zoonotic potential; therefore, the relatively high prevalence of infection in the examined children is probably a result of low hygienic standards and exposure to animals.

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