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Diversity of microsporidia (Fungi: Microsporidia) among captive great apes in European zoos and African sanctuaries: evidence for zoonotic transmission?

Bohumil Sak[1], **Martin Kváč**[1,2], **Klára Petrželková**[3,4], **Dana Květoňová**[1], **Kateřina Pomajbíková**[5], **Martin Mulama**[6], **John Kiyang**[7] and **David Modrý**[1,5]

[1]Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, Branišovská 31, České Budějovice, Czech Republic;

[2]Faculty of Agriculture, University of South Bohemia in České Budějovice, Czech Republic;

[3]Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, Brno, Czech Republic;

[4]Liberec Zoo, Liberec, Czech Republic;

[5]Department of Parasitology, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic;

[6]Sweetwaters Chimpanzee Sanctuary, Nanyuki, Kenya;

[7]Limbe Wildlife Centre, Cameroon

Abstract: Two hundred and seventeen captive great apes (150 chimpanzees, *Pan troglodytes*; 14 bonobos, *Pan paniscus*; 53 western gorillas, *Gorilla gorilla*) and 20 personnel from thirteen European zoos and two African sanctuaries were sampled and examined in order to determine the occurrence of *Enterocytozoon bieneusi* and species of *Encephalitozoon* in faecal specimens and to compare the epidemiological situation between zoos and sanctuaries. Microsporidia were detected at all sampling sites. Sequence analyses of ITS amplicons generated by using microsporidia-specific primers determined the presence of microsporidia in 87 samples including 13 humans; since two cases of simultaneous occurrence of *Encephalitozoon cuniculi* and *Enterocytozoon bieneusi* were identified, 89 full-length ITS sequences were obtained, namely 78 *Encephalitozoon cuniculi* genotype I, five *E. cuniculi* genotype II, two *E. hellem* 1A and four *Enterocytozoon bieneusi.* No *Encephalitozoon intestinalis*-positive samples were identified. This is the first report of *Encephalitozoon* species and *Enterocytozoon bieneusi* genotypes in captive great apes kept under various conditions and the first record of natural infection with *E. hellem* in great apes. A comparison of zoos and sanctuaries showed a significantly higher prevalence of microsporidia in sanctuaries ($P<0.001$), raising a question about the factors affecting the occurrence of microsporidia in epidemiologically and sanitarily comparable types of facilities.

Keywords: *Enterocytozoon bieneusi*, *Encephalitozoon*, prevalence, primates, chimpanzee, *Pan troglodytes*, bonobo, *Pan paniscus*, western gorilla, *Gorilla gorilla*

Microsporidia are eukaryotic, intracellular parasitic fungi that infect a wide range of invertebrate and vertebrate hosts including both humans and non-human primates. They are considered to be one of the most common parasitic protists of mammals, producing the infections ranging in clinical severity from asymptomatic to highly pathogenic or even lethal ones, mainly in immunocompromised individuals (Gannon 1980, Weber et al. 1994, Didier et al. 2004).

Microsporidia of the genera *Enterocytozoon* and *Encephalitozoon* have frequently been reported in various domestic and free-living animals (Deplazes et al. 1996, Bornay-Llinares et al. 1998, Kašičková et al. 2009, Santín and Fayer 2009). However, there is only a single study on microsporidia in great apes (Graczyk et al. 2002) despite the fact that African great apes are undoubtedly our closest extant relatives (Kumar et al. 2005) and that great attention is currently paid to microsporidia and microsporidiosis in humans (i.a. Mathis et al. 2005).

In the recent past, increased human population density and reduction and fragmentation of natural habitats have increased the intensity and intimacy of human-primate contacts to the level greater than at any time during the primate evolution. The novel epidemiological situation has undoubtedly increased the exchange of pathogens between primates, humans and livestock (Goldberg et al. 2008, Köndgen et al. 2008, Rwego et al. 2008, Kaur et al. 2008). Especially under captive conditions, the primates

Address for correspondence: M. Kváč, Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, Branišovská 31, 370 05 České Budějovice, Czech Republic. Phone: +420 38 777 5419; Fax: +420 38 5310388; E-mail: kvac@paru.cas.cz

come to direct contact with people and indirect contact with numerous exotic mammalian species, which offers a plethora of opportunities for the transmission of pathogens including microsporidia (Mathis et al. 2005).

This study was aimed to assess the diversity of microsporidia in captive great apes and to compare their occurrence in two comparable types of facilities: in European zoos and African sanctuaries. Additionally, we report on microsporidia infections of humans working in close contact with captive primates.

MATERIALS AND METHODS

Sample collection. From 2005 to 2009, we collected 217 fresh faecal samples from 150 chimpanzees (*Pan troglodytes*), 14 bonobos (*Pan paniscus*) and 53 western gorillas (*Gorilla gorilla*) from 13 zoos in 10 countries across Europe and from two sanctuaries in Africa, namely Limbe Wildlife Centre in Cameroon and Sweetwaters Chimpanzee Sanctuary in Nanyuki, Kenya (Table 1). Additionally, we obtained 20 stool samples of animal keepers and other humans working in Limbe Wildlife Centre as a part of employees' health monitoring (Table 1). Participants were acquainted with the study and oral consent to participate in the study was obtained. Samples were preferably taken from identified animals; however, in some facilities individual sampling was impossible. Even in these cases we tried our best to prevent repeated sampling of the same individual. At the time of collection, the animals and people were clinically healthy. Part of each faecal sample was immediately preserved in 96% ethanol and later transported to the Institute of Parasitology, Biology Centre of Academy of Sciences of the Czech Republic. The collection of faecal samples of great apes was non-invasive and did not cause any disturbance of the animals. Research adhered to the legal requirements of the countries in which it was conducted.

DNA isolation. Faecal samples were homogenized by bead disruption using a FastPrep®-24 Instrument (MP Biomedicals, CA, USA) and 0.5 mm glass beads (Biospec Products, Inc., Bartlesville, OK, USA) at the speed of 5 m/s for 1 min. Total DNA was extracted using a commercial column based isolation kit QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Acquired DNA was stored at −20°C.

PCR amplification. We used microsporidia-specific primers described by Katzwinkel-Wladarsch et al. (1996). The upstream primers MSP-1 (TGA ATG (G,T)GT CCC TGT) and MSP-3 (GGA ATT CACACC GCC CGT C(A,G)(C,T) TAT) were targeted to the 3' region of the SSU coding segment of *Enterocytozoon bieneusi* and all three species of *Encephalitozoon*. The downstream primers MSP-2B (GTT CAT TCG CAC TAC T) and MSP-4B (CCA AGC TTA TGC TTA AGT CCA GGG AG) were targeted to the 5' region of the LSU coding segment of *E. bieneusi*. The downstream primers MSP-2A (TCA CTC GCC GCT ACT) and MSP-4A (CCA AGC TTA TGC TTA AGT (C,T) (A,C)A A(A,G)G GGT) were targeted to the 5' region of the coding segment of species of *Encephalitozoon*. For the primary PCR step, the PCR mixture contained 1× PCR buffer, 3 mM $MgCl_2$, 0.2 mM each dNTP's, 1 U *Taq*, 1 µl BSA (10 mg/ml), and 200 nM each primer. For the secondary PCR step, the PCR mixture was identical except that BSA was excluded. For both PCR steps a total of 35 cycles, each consisting of 94 °C for 45 s, 54 °C for 45 s, and 72 °C for 60 s, were performed. Initial incubation at 94 °C for 3 min., final extension at 72 °C for 7 min., and final soak at 4 °C were included. PCR products were visualized on a 2% agarose gel containing 0.2 µg/ml ethidium bromide.

PCR products were directly sequenced on an ABI3730XL sequence analyzer (Applied Biosystems, Foster City, CA). Each sample was sequenced in both directions. Sequences were aligned and completed using the programmes ChromasPro (Technelysium, Pty, Ltd., version 1.5), BioEdit sequence alignment software version 7.0.5.3. (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and Clustal X (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/, version 2.0.12) and compared with sequences in GenBank.

DNA from *E. intestinalis* spores originally isolated from an AIDS patient (Didier et al. 1996a) and grown *in vitro* in VERO E6 cells in the Laboratory of Veterinary and Medical Protistology at the Institute of Parasitology of ASCR and from *E. bieneusi* spores of genotype D originally isolated from a pig (Sak et al. 2008) was used as positive control.

Statistical analyses. The Chi-square test was used to evaluate differences in the prevalence of microsporidia between sanctuaries and zoos. Statistical tests were calculated using Statistica1, Release 5.1 Software (Statsoft, Tulsa, OK, USA, 1997).

RESULTS

Out of the total of 237 examined samples, 87 (36.7%) were tested positive for the presence of microsporidia DNA by nested PCR with genus-specific primer sets. Since two cases of simultaneous occurrence of *Encephalitozoon cuniculi* and *Enterocytozoon bieneusi* were identified, 89 full-length ITS sequences were obtained, namely 85 of species of *Encephalitozoon* and 4 of *Enterocytozoon bieneusi*. The alignment of the obtained sequences with reference ITS sequences showed 100% homology with GenBank-listed species and their genotypes as follows: 78 *E. cuniculi* genotype I (AF338410), 5 *E. cuniculi* genotype II (GQ422153) and 2 *E. hellem* genotype 1A (AF338367). Sequence analyses of the four samples positive for *E. bieneusi* revealed the presence of three different genotypes (Table 1). The alignment of these sequences with ITS sequences listed in GenBank demonstrated 100% identity as follows: EbpA (AF076040), PigEBITS5 (AF348473) and D (AF101200).

Single-species infection was detected in most animals (number of animals in parentheses): *E. cuniculi* genotype I (63), *E. cuniculi* genotype II (5), *E. hellem* 1A (2) and *E. bieneusi* (2). Co-infection was detected in one chimpanzee in Limbe Wildlife Centre (*E. cuniculi* I together with *E. bieneusi* genotype PigEBITS5) and one chimpanzee in Zoo Bratislava (*E. cuniculi* I together with *E. bieneusi* genotype D).

The overall microsporidia prevalence in sanctuaries was significantly higher compared to zoos (53.1% vs. 20.2%; $P<0.001$; $\chi^2=25.55$). This trend was significant even if we compared individual host species separately (chimpanzees: 44.6% vs. 22.2%, $P<0.01$; $\chi^2=9.19$; gorillas: 100.0% vs. 15.8%, $P<0.001$; $\chi^2=31.88$). No

Table 1. Molecular detection of microsporidia species/genotypes in primates from captive facilities and in humans working in sanctuaries. *E.c.*I – *Encephalitozoon cuniculi* genotype I; *E.c.*II – *E. cuniculi* genotype II; *E.h.* – *E. hellem* genotype 1A; *E.b.* – *Enterocytozoon bieneusi*. Genotypes of *E. bieneusi*: [a]EpbA, [b]PigEBITS5, [c]D; *dual infection with *E. cuniculi* and *E. bieneusi*; BPRC – Biomedical Primate Research Centre; LWC – Limbe Wildlife Centre; SWS – Sweetwaters Sanctuary.

Location (country)		*Pan troglodytes*				*Pan paniscus*	*Gorilla gorilla*	*Homo sapiens*	TOTAL
		*E.c.*I	*E.c.*II	*E.h.*	*E.b.*	*E.c.*I	*E.c.*I	*E.c.*I	
		positive/examined							
Sanctuary	LWC (CM)	19*/44	3/44	1/44	2[a*,b]/44	–	15/15	13/20	53/79
	SWS (KE)	9/39	1/39	1/39	1[c]/39	–	–	–	12/39
Zoo	Amersfoort (NL)	0/4	0/4	0/4	0/4	–	–	–	0/4
	Antwerp (B)	0/5	0/5	0/5	0/5	0/5	0/1	–	0/11
	Rijswijk BPRC (NL)	2/12	0/12	0/12	0/12	–	–	–	2/12
	Bratislava (SK)	2*/3	0/3	0/3	1[c]*/3	–	–	–	3/3
	Dublin (IR)	4/9	0/9	0/9	0/9	–	0/3	–	4/12
	La Palmyre (F)	0/3	0/3	0/3	0/3	–	0/4	–	0/7
	La Vallée des Singes (F)	0/5	0/5	0/5	0/5	–	1/6	–	1/11
	Leipzig (D)	2/5	0/5	0/5	0/5	1/5	2/5	–	5/15
	Madrid (E)	2/9	0/9	0/9	0/9	–	0/5	–	2/14
	Opole (PL)	–	–	–	–	–	1/3	–	1/3
	Pilsen (CZ)	1/5	1/5	0/5	0/5	–	–	–	2/5
	Prague (CZ)	–	–	–	–	–	2/5	–	2/5
	Twycross (UK)	0/7	0/7	0/7	0/7	2/4	0/6	–	2/17
TOTAL		41/150	5/150	2/150	4/150	3/14	21/53	13/20	89/237

differences were found among individual zoos or between both sanctuaries.

While *E. cuniculi* genotype I was found in all host species examined including employees from Limbe Wildlife Centre (87.6% of all positive samples), *E. cuniculi* genotype II, *E. hellem* 1A and *E. bieneusi* were found only in chimpanzees, mostly originating from sanctuaries. The great majority of *E. cuniculi* genotype I cases was detected in animals kept in sanctuaries (43.8% vs. 18.5%, $P<0.001$; $\chi^2=16.51$), predominantly in chimpanzees and gorillas in Limbe Wildlife Centre. In addition, a high occurrence of *E. cuniculi* genotype I was recorded among employees from Limbe Wildlife Centre (65.0%) (Table 1).

DISCUSSION

Hominids, including humans, share not only the common evolutionary history but also several pathogens that are able to cross the species boundaries (Wolfe et al. 1998, Leroy et al. 2004, Keele et al. 2006, Davies and Pedersen 2008). Undoubtedly, the increased contact between apes and humans in recent years has aggravated the risk of transmission of a plethora of zoonotic infectious diseases (Kaur et al. 2008, Köndgen et al. 2008, Johnston et al. 2010), possibly including microsporidioses.

In our study, focusing on captive chimpanzees and gorillas from various facilities around Europe and Africa, we documented the presence of three species and six genotypes of microsporidia from the genera *Encephalitozoon* and *Enterocytozoon*.

Although various microsporidia species and genotypes have been reported in humans repeatedly (Mathis et al. 2005), infections of non-human primates have been documented rarely (Wasson and Peper 2000). However, natural occurrence of microsporidia in captive non-human primates has been recorded in members of both Strepsirrhini (Slodkowitcz-Kowalska et al. 2007) and Haplorrhini (van Dellen et al. 1989, Schwartz et al. 1998, Wenker et al. 2002, Reetz et al. 2004, Asakura et al. 2006, Juan-Sallés et al. 2006, Davis et al. 2008). *Encephalitozoon cuniculi* (genotypes II and III) has been revealed as a major causative agent of microsporidioses in monkeys, in some cases with fatal consequences (Wenker et al. 2002, Guscetti et al. 2003, Reetz et al. 2004, Davis et al. 2008). The high prevalence of microsporidia, especially of *E. cuniculi*, among the apes included in our study corresponds not only with these data but also with its common occurrence among the humans (Rossi et al. 1998, Snowden et al. 1999, Tosoni et al. 2002) and other animals (Deplazes et al. 1996, Snowden et al. 1999). On the other hand, spontaneous infection with *E. bieneusi* and *E. intestinalis* in non-human primates has been recorded only sporadically (Schwartz et al. 1998, Graczyk et al. 2002, Slodkowicz-Kowalska et al. 2007), which is in agreement with our results; we detected only four cases of *E. bieneusi* infection and none of *E. intestinalis*. So far, no *E. hellem* infections have been reported in any non-human primates. Despite the fact that *E. hellem* is not rare in the human population (Xiao et al. 2001, Haro et al. 2003), its detection in two chimpanzees from Limbe Wildlife Centre and Sweetwaters Sanctuary is noteworthy.

Discrimination of the genotypes is crucial for uncovering the infraspecific diversity of microsporidia, but mainly for unravelling the sources of infection and transmission patterns. All microsporidia species/genotypes identified in our study were previously found in different avian

and mammalian hosts including humans. The source of spores of the reported microsporidia genotypes remains speculative because of ubiquitous occurrence, low host specificity and long-term resistance of spores in different environmental conditions (Cotte et al. 1999, Thurston-Enriquez et al. 2002, Didier et al. 2004, Mathis et al. 2005). Based on molecular data of ITS rDNA, three *E. cuniculi* strains have been identified, strain I ("rabbit"), strain II ("mouse") and strain III ("dog") (Didier et al. 1995). All these genotypes have been found to infect humans, implying the zoonotic nature of the infection (Deplazes et al. 1996, Didier et al. 1996b, Rinder et al. 1998, Rossi et al. 1998, Snowden et al. 1999, Tosoni et al. 2002). *Encephalitozoon cuniculi* genotypes I and II were identified in our set of faecal or stool samples. The high prevalence of *E. cuniculi* genotype I among both animal keepers and apes in Limbe Wildlife Centre implies the potential human to ape or *vice versa* transmission.

The intraspecific variability of *E. hellem* is based on differences in ITS and polar tube protein (PTP) gene sequences, resulting in five different genotypes named 1A, 1C, 1D, 2B and 2C (Haro et al. 2003). *Encephalitozoon hellem* genotype 1A detected in two chimpanzees in our study has been recorded only in birds so far (Suter et al. 1998, Snowden et al. 2000, 2001, Haro et al. 2005) except for one case of human infection in Spain (Xiao et al. 2001).

On the contrary, *Enterocytozoon bieneusi* is a complex species with multiple genotypes and diverse host range. On the basis of PCR analysis of the ITS rRNA gene, 81 different genotypes of *E. bieneusi* have been described in humans and animals. Twenty-six of those genotypes have been found exclusively in humans, and eight different genotypes have been reported in humans and animals (Santín and Fayer 2009). We identified three different genotypes, namely EbpA, D, and PigEBITS5, among the four *E. bieneusi*-positive samples. These genotypes were previously described in cattle (EbpA, D), pigs (D, PigEBITS5, EbpA) and humans (all of them) (del Aguila et al. 1999, Deplazes et al. 2000, Lores et al. 2002, Sulaiman et al. 2003a, b).

The observed difference in the occurrence of microsporidia between sanctuaries and zoos is noteworthy, though difficult to explain. In our opinion, the overall epidemiological situation and level of sanitation in European zoos and two monitored African sanctuaries are comparable. The close human-ape contact could contribute to the transmission of microsporidia infections from asymptomatic human hosts, however, the prevalence of microsporidiosis seems to be similar among people living in Africa and Europe (Abreu-Acosta et al. 2005, Mungthin et al. 2005, Tumwine et al. 2005, Nkinin et al. 2007, Samie et al. 2007). Then, different environmental conditions and climate might offer the most plausible explanation. Generally, spores of *Encephalitozoon* spp. could be shed during systemic infections beside faeces also in urine (e.g. Sak et al. 2011). Nevertheless, the urine testing was not performed due to impossibility to obtain uncontaminated sterile urinal samples in the present study, therefore possibility that some animals were positive in urine and not in faecal samples remains speculative.

Similarly to the healthy human population (Abreu-Acosta et al. 2005, Nkinin et al. 2007), neither apes nor animal keepers included in this study exhibited clinical signs of microsporidiosis (e.g. loose stool). Interestingly, in the New World monkeys, such as squirrel monkeys *Saimiri sciureus*, emperor tamarins *Saguinus imperator* and golden lion tamarins *Leontopithecus rosalia*, severe cases of encephalitozoonosis with high morbidity and mortality have been described (Deplazes et al. 2000, Guscetti et al. 2003), implying unexplained elevated susceptibility to the infection.

To our knowledge, this is the first study using molecular tools providing detailed information about the occurrence of microsporidia in great apes. Numerous recent studies showed the broad host specificity of microsporidia of the genera *Encephalitozoon* and *Enterocytozoon*. The presence of *E. bieneusi*, *E. cuniculi* and *E. hellem* detected in captive great apes in our study is in agreement with the nearly ubiquitous character of these pathogens. Our results from captive apes indicate that the close human-ape contact might be important for the occurrence of microsporidia even in the wild. However, only the thorough examination of sets of samples from wild great apes with different level of contact with humans and from habitats with different level of anthropogenic disturbance can answer the question about the importance of human-ape contact for the epidemiology of microsporidia infections in great apes.

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