IS MICROSPIRIDIAL INFECTION IN ANIMALS A POTENTIAL SOURCE FOR HUMAN MICROSPORIDIOSIS?

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Abstract. Microsporidiosis is an emerging infectious disease among a rapidly-broadening clinical spectrum of diseases that can cause significant morbidity in immunocompromized and immunocompetent patients, especially in patients with acquired immunodeficiency syndrome (AIDS). Acquired infection seems to occur from person-to-person transmission or from other sources (*eg* water, food, and animals). Several microsporidia species that are pathogenic for humans have been identified in domestic and wild animals. The aims of this study were to determine the presence of microsporidian spores in stool samples from animals and to identify the species implicated in infection, in Portugal. A total of 352 stool samples from animals (117 pets, 99 animals from the Zoo, 51 bovines, and 85 sylvatic animals) were studied. Modified trichrome stain, IFA with MAb 3B6 and PCR with species-specific primers for *Enterocytozoon bieneusi, Encephalitozoon intestinalis, E. cuniculi,* and *E. hellem* SSU-rRNA gene followed by nucleotide sequencing were the methods used. Microsporidian spores were identified in stools from 32 (27%) pets and 15 (15%) animals from the Zoo, presenting 38 (81%) of these animals with low to moderate parasite loads. All stool samples from small rodents were negative for microsporidia by MT and IFA. By PCR, 13 isolates (4 from cats, 3 from dogs, 1 from a white-fronted marmoset, and 5 from bovines) were identified as being *E. bieneusi*-positive and confirmed by sequencing. In conclusion, animals in close contact with humans harbor microsporidia species also identified in humans, releasing spores into the environment with feces that can be a source of human infection. This study sets the framework for further studies on the epidemiology of microsporidia infection in Portugal.

INTRODUCTION

Microsporidia are obligate intracellular protozoan parasites infecting a broad range of vertebrates and invertebrates. During the past decade, several genera of microsporidia have been identified in immunocompromized patients, especially those with acquired immunodeficiency syndrome (AIDS) (Weiss, 2001), and more recently in immunocompetent persons (Weiss, 2001; Matos *et al*, 2001, 2002; Lobo *et al*, 2002). Microsporidia can cause a wide spectrum of intestinal or disseminated pathologies depending on the infectious species involved and also on the immune status of the host. Routes of transmission and sources of human microsporidial infections have been difficult to ascertain. Acquired infection seems to occur from person-to-person transmission or from other sources (*eg* water, food, and animals).

Several microsporidia species pathogenic to humans have been identified in domestic and wild animals (Wright and Craighead, 1922; Levaditi *et al*, 1923; Didier *et al*, 1995; Deplazes *et al*, 1996a,b; Mathis *et al*, 1996, 1999; Black *et al*, 1997; Mansfield *et al*, 1997; Bornay-Llinares *et al*, 1998; Pulparampil *et al*, 1998; Breitenmoser *et al*, 1999; Del Aguila *et al*, 1999; Rinder *et al*, 2000; Snowden *et al*, 2000; Dengjel *et al*, 2001; Buckholt *et al*, 2002; Graczyk *et al*, 2002; Lores *et al*, 2002; Muller- Doblies *et al*, 2002; Fayer *et al*, 2003; Lobo *et al*, 2003; Sulaiman *et al*, 2003). Although this fact supports the hypothesis that human infections may be a zoonosis, direct evidence of transmission from animals to humans is still lacking. Persons or animals infected with these parasites release spores into the environment through feces, urine or respiratory secretions that may be potential sources of infection.

In Portugal, 51, 18 and 7% of microsporidian spores were detected, respectively, in feces, pulmonary specimens, and urine of AIDS patients (Matos *et al*, 2001, 2002), and 46% in feces from immunocompetent individuals (Lobo *et al*, 2002). The great majority of the intestinal infections were symptomatic, ranging from gastrointestinal complaints without diarrhea, transient diarrhea (in immunocompetent patients) to chronic diarrhea (in immunocompromized patients).

This study aimed to identify potential animal sources (domestic and sylvatic animals) of human

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microsporidial infection and to determine the microsporidia species isolated from human and animal samples.

MATERIALS AND METHODS

A total of 352 stool samples from animals of veterinary clinics (117 pets – 58 dogs, 58 cats, and one parrot) in Lisbon, 99 animals from the Lisboa Zoo (primates, ruminants, reptiles, birds, and marsupials), 51 bovines from central and southern Portugal, and also from the Azores Islands, and 85 small rodents from southern Portugal were examined for the presence of microsporidian spores. All samples, except the bovine ones [studied only by polymerase chain reaction (PCR)], were analyzed by modified trichrome staining (MT) and monoclonal antibody (MAb) 3B6-based immunofluorescence assay (IFA) to identify microsporidian spores. The number of spores presented in each stool sample was determined semi-quantitatively, based on the following scoring system: the number of spores counted per microscopic field (under a 100× objective) + (one to five spores), ++ (six to 10 spores), +++ (11 to 15 spores), and ++++ (>15 spores).

Specimen processing

Spores were concentrated from fecal material by modified water-ether sedimentation method. Briefly, 2 ml feces were vigorously mixed with 2 ml ether and 5 ml distilled water, filtered through gauze and centrifuged at 4,100*g* for 20 minutes. The upper phase was discarded and the supernatant was recovered, while the sediment was kept. The recovered supernatant was centrifuged again at 4,100*g* for 15 minutes and the sediment obtained was added to the one kept previously. The combined sediments were washed three times with water, centrifuged for 15 minutes at 4,100*g* after each wash, and resuspended in 1 ml water before storage at -20˚C. From each stool sample, one smear from the sediment obtained was made on two glass microscope slides and each slide was stained by the two methods. All sediments were stored at -20˚C for later analysis.

The two techniques were performed without knowledge of either results or the animals' clinical data.

Staining methods

The modified trichrome stain originally used for microsporidia (del Aguila *et al*, 2001), adapted for this study, and the IFA-MAb 3B6 staining method were performed as previously described (Enriquez *et al*, 1997; Matos *et al*, 2001, 2002).

A sample was considered positive when both staining techniques presented concordant positive results.

DNA extraction

DNA isolation was performed by a Mini-BeadBeater / silica method. Briefly, 400 µl of concentrated spores were added to 900 µl lysis buffer [7 M guanidium thiocyanate, 50 mM Tris-HCl (pH 6.4), 25 mM EDTA (pH 8.0) and 1.5% (v/v) Triton X-100], 60 µl isoamyl alcohol and 0.3 g 0.5-mm-diameter zirconia beads (Stratech Scientific, Luton, UK). The tube was shaken in a Mini-BeadBeater (Stratech Scientific) at maximum speed for 2 minutes, left at room temperature for 5 minutes, and centrifuged in a micro-centrifuge (at full speed for 15 seconds) to pellet the zirconia beads and the fecal debris. A 40 µl volume of a suspension of coarse, activated silica (1%, w/v; pH 2.0) was then added to the supernatant solution and incubated at room temperature for 60 minutes under gentle agitation. After a short centrifugation (at full speed for 15 seconds in a microcentrifuge) and removal of the supernatant, the silica pellet was washed twice with 200 µl wash buffer (7 M guanidium thiocyanate, 50 mM Tris-HCl (pH 6.4), once with 200 µl 80% ethanol and once with 200 µl acetone. The pellet was then dried at 55˚C for 10 minutes before the DNA was eluted by resuspension of the pellet in 60 µ l sterile distilled water and incubation at 55˚C for 10 minutes. After centrifugation at 23,100*g* for 2 minutes, the supernatant with the DNA was recovered and stored at -20˚C for further use.

PCR amplification and DNA sequencing

PCR primers were chosen to amplify a conserved region of the small-subunit (SSU) rRNA gene of four microsporidia reportedly pathogenic to humans. The four primer sets EBIEF1/EBIER1 (Da Silva *et al*, 1996), SINTF1/SINTR1 (Da Silva *et al*, 1997), ECUNF1/ECUNR1 (Visvesvara *et al*, 1994) and EHELF1/EHELR1 (Visvesvara *et al*, 1994) produced fragments of 607 bp, 520 bp, 549 bp, and 546 bp, from *Enterocytozoon bieneusi, Encephalitozoon intestinalis, Encephalitozoon cuniculi,* and *Encephalitozoon hellem* SSU-rRNA, respectively. PCRs with these species-specific primers were performed with all positive samples by the staining methods. PCR products were analyzed on 2% agarose gel electrophoresis and ethidium bromide, were purified using a Jetquick kit (Genomed), and further resuspended in sterile H_20. PCR sequencing was performed on a Beckman Coulter CEQ 2000XL.

SSU-rRNA sequence analysis

Database searching was performed with BLAST

2.0 on the National Center for Biotechnology Information's World Wide Web site (http://www.ncbi.nlm.nih.gov).

RESULTS

MT and IFA identified microsporidian spores in stools samples from 32 (27%) pets and 15 (15%) animals from the Zoo (Table 2), presenting 26 (55%) low parasite load (+), 12 (26%) moderate load (++), and nine (19%) high load (+++). All stool samples from small rodents were negative for microsporidia by MT and IFA. Microsporidian spores stained by MT appeared as rod-shaped or ovoid (mainly in samples from the Zoo animals) structures stained pinkish-red with a spore wall and the characteristic equatorial belt-like stripe, or displayed an apple-green fluorescence when stained by IFA-Mab 3B6. By PCR, 13 isolates (4 from cats, 3 from dogs, 1 from a white-fronted marmoset, and 5 from bovines) were identified *E. bieneusi*-positive by the presence of PCR products of the expected size, 607 bp (Table 1). To avoid PCR inhibitors in all samples, serial 10-fold template dilutions were prepared. No amplification was detected in the remaining 34 samples, when the four species-specific primers were used. For the samples positive by staining method, two interpretations may be considered: stool samples contained DNA polymerase inhibitors that were not eliminated with dilutions, or the amount of DNA was below the limit of detection. All isolates amplified by PCR were sequenced. The sequence was then entered into the National Center for Biotechnology Information's BLAST 2.0 search engine and subjected to database homology testing. The sequence of amplified DNA fragments showed 100% homology with the sequences of GenBank database accession numbers AF023245, AF024657, L16868 (*E. bieneusi*), confirming the results obtained by PCR.

DISCUSSION

Microsporidiosis is an emerging infectious disease among a rapidly broadening clinical spectrum of diseases that can cause significant morbidity, especially in immunocompromized patients. Since 1995, several studies have reported identification of microsporidia in animals. The use of molecular tools with a greater capacity to detect and differentiate species and strains has resulted in identifying species infective for humans in the feces of animals in close contact with humans (Wright and Craighead, 1922; Levaditi *et al*, 1923; Didier *et al*, 1995; Deplazes *et al*, 1996a; Mathis *et al*, 1996, 1999; Mansfield *et al*, 1997; Black *et al*, 1997; Pulparampil *et al*, 1998; Bornay-Llinares *et al*, 1998; Breitenmoser *et al*, 1999; del Aguila *et al*, 1999; Snowden *et al*, 2000; Rinder *et al*, 2000; Dengjel *et al*, 2001; Buckholt *et al*, 2002; Graczyk *et al*, 2002; Lores *et al*, 2002; Muller-Doblies *et al*, 2002; Fayer *et al*, 2003; Lobo *et al*, 2003; Sulaiman *et al*, 2003)

Table 1
Detection of microsporidia infection among 352 animals, in Portugal.

Animals studied (No.)	Positive by staining method	Positive by PCR			
	No. (%)	*E. bieneusi*	*E. intestinalis*	*E. cuniculi*	*E. hellem*
Pets (117)					
Cats (58)	22 (38)	4	0	0	0
Dogs (58)	9 (16)	3	0	0	0
Parrot (1)	1 (100)	0	0	0	0
Zoo animals (99)	15 (15)	1	0	0	0
Sylvatic animals (small rodents) (85)	0 (0)	ND	ND	ND	ND
Bovines (51)	ND	5	0	0	0
Total (352)	47	13	0	0	0

ND- Not determined

Table 2
Summary of animal reservoirs for microsporidia infecting humans in published studies.

Host	Species detected in animals	References
Rabbits	*Encephalitozoon cuniculi*	Wright and Craighead (1922); Levaditi *et al* (1923)
	Encephalitozoon cuniculi	
Rabbits (Switzerland, USA)	Strain I	Didier *et al* (1995); Deplazes *et al* (1996)
Mouse (Czech Rep, UK) Blue foxes (Norway)	Strain II	Didier *et al* (1995) Mathis *et al* (1996)
Dogs (USA, South Africa)	Strain III	Didier *et al* (1995)
Pigs (Switzerland) Monkeys (USA)	*Enterocytozoon bieneusi*	Deplazes *et al* (1996a) Mansfield *et al* (1997)
Psittacine birds: Budgerigars (USA) Parrots (USA)	*Encephalitozoon hellem*	 Black *et al* (1997) Pulparampil *et al* (1998)
Donkeys, dogs, pigs, cow, goat (Mexico)	*Encephalitozoon intestinalis*	Bornay-Llinares *et al*(1998)
Rabbits, dogs (Spain)	*Enterocytozoon bieneusi*	Del Aguila *et al* (1999)
Dogs, cat (Switzerland)	*Enterocytozoon bieneusi*	Mathis *et al* (1999)
Pigs (Switzerland)	*Enterocytozoon bieneusi*	Breitenmoser *et al* (1999)
Peach-faced lovebird (USA)	*Encephalitozoon hellem*	Snowden *et al* (2000)
Cattle (Germany)	*Enterocytozoon bieneusi*	Rinder *et al* (2000)
Pig, cattle, cats, llama (Germany)	*Enterocytozoon bieneusi*	Dengjel *et al* (2001)
Dogs, goat (Spain)	*Enterocytozoon bieneusi*	Lores *et al* (2002)
Pigs (USA)	*Enterocytozoon bieneusi*	Buckholt *et al* (2002)
Gorillas (Uganda)	*Encephalitozoon intestinalis*	Graczyk *et al* (2002)
Free-ranging rat (Switzerland))	*Encephalitozoon cuniculi*	Muller- Doblies *et al* (2002)
Calves (USA)	*Enterocytozoon bieneusi*	Fayer *et al* (2003)
Beavers, foxes, muskrats, otters, and raccoons (USA)	*Enterocytozoon bieneusi*	Sulaiman *et al* (2003)
Dogs, cats (Portugal)	*Enterocytozoon bieneusi*	Lobo *et al* (2003)

(Table 2). The identification of potential animal sources of human microsporidial infection seems relevant.

The results of this study indicated a high frequency of microsporidian spores in animals in close contact with humans (27% in pets, 15% in animals from the Zoo, and 15% in bovines), being *E. bieneusi*, until now the only species detected and confirmed by sequencing in stool samples from animals in Portugal. This study also showed that animals can be infected with microsporidia species normally infective for humans.

Parasitologically, infections in animals with microsporidia had low to moderate spore shedding (81% of the animals parasitized had low to moderate parasite loads). The majority of positive samples was from animals without diarrhea, suggesting that infections were asymptomatic.

In conclusion, a larger sample size needs to be studied and comparative analysis of the species and strains of microsporidia isolated from humans and animals needs to be done. The identification of microsporidian spores in animals that can be a possible source of infection for humans may reduce and prevent human exposure, mainly in groups of people at risk of microsporidiosis (*eg* immunocompromized, children, the elderly).

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