Enterocytozoon bieneusi (Microsporidia) in Faecal Samples from Domestic Animals from Galicia, Spain

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In this survey we examined 87 domestic animal stool samples in order to detect the possible presence of microsporidia in animals in close contact with humans in Galicia (NW, Spain). The detection of Enterocytozoon bieneusi *spores was confirmed in faecal samples from two dogs and one goat by polymerase chain reaction. None of the positive samples for microsporidia in the staining method were amplified with species-specific primers for* Encephalitozoon intestinalis, E. hellem *and* E. cuniculi. *Four rabbits faecal samples reacted with anti-*E. cuniculi *serum. Our results could indicate the importance of domestic animals as zoonotic reservoirs of microsporidial human infections.*

Key words: *Enterocytozoon bieneusi* - domestic animals - faeces - zoonotic origin - Galicia - Spain

Microsporidia are protozoan parasites belonging to the phylum Microsporidia within which exist over 1000 species classified into approximately 100 genera. These eukaryotic obligate intracellular protozoans have been described infecting every major animal group, especially insects, fish, and mammals (Wittner 1999). Microsporidia have been increasingly recognized as opportunistic pathogens of immunodeficient patients (Weber et al. 1994), especially in Aids patients but it is also becoming increasingly common in immunocompetent individuals (Gainzarain et al. 1998, Lores et al. 2001).

Although during the last decade numerous data related to the epidemiology of this infection in humans and animals have been accumulated, implying a zoonotic nature of these parasites, direct evidence of transmission from animals to humans are still lacking (Deplazes et al. 2000).

Encephalitozoon cuniculi is probably the most extensively studied mammalian microsporidian and has been reported to infect a wide range of hosts, including common laboratory rodents as well as human and non-human primates. This is the first microsporidian species infecting humans that has been considered a zoonosis (Deplazes et al. 1996, Didier et al. 1996) .

The first identification of *E. intestinalis* in mammals other than humans was reported by Bornay et al. (1998) in the faeces of donkeys, dogs, pigs, cow, and goat suggesting that *E. intestinalis* might also be of zoonotic origin.

Enterocytozoon bieneusi is the most frequent microsporidian found in humans, especially in Aids patients. It has been associated mainly with chronic diarrhoea, although it has been diagnosed in patients with other forms of immunosuppression and in immunocompetent travellers with self-limited diarrhoea (Weber & Bryan 1994, Sobottka et al. 1995). In addition, this pathogen has recently been detected in other natural hosts such as pigs (Deplazes et al. 1996, Breitenmoser et al. 1999, Rinder et al. 2000), cows, goats, pigs, chickens, cats, turkeys (Bornay et al. 1998), rabbits, dogs (del Aguila et al. 1999), and in simian immunodeficiency virus-inoculated monkeys (Tzipori et al. 1997, Mansfield et al. 1997). Consequently, this microsporidian infection may be more common than previously suspected.

In this study, we used microscopic, immunologic, and molecular methods to detect microsporidial spores in faecal samples of domestic animals from Galicia (NW, Spain). We have designed this survey in order to expand our knowledge concerning the pathogenic role of microsporidia in animals having close contact with humans.

MATERIALS AND METHODS

Animal stool samples - A total of 87 faecal samples from 75 domestic animals (22 rabbits, 17 dogs, 10 cats, 4 pigs, 2 donkeys, 10 horses, 7 goats, and 3 cows) were collected during a study conducted in rural villages in the provinces of Pontevedra and Ourense (NW, Spain). Samples were stored in 10% formalin for several months. At the time of this study, samples were concentrated by ethyl acetate, and stored at 4ºC until used. Unpreserved samples were aliquoted and conserved at -80ºC for molecular study.

Light microscopy - The samples were stained with the Weber's chromotrope to investigate microsporidia as described (Weber et al. 1992). A search for ova and parasites was carried out, including ethyl acetate sedimentation, Ziehl-Neelsen stain and a specific monoclonal antibody test for *Cryptosporidium* species (Merifluor®, Meridian Diagnostics, Inc.).

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Microorganisms - *E. cuniculi* (ECLD), *E. hellem* (CDC:V257) and *E. intestinalis* (CDC:V297), were cultured on E6 monolayers to be used as controls (Visvesvara et al. 1995).

Indirect immunofluorescence test (IIF) - Spores of *E. cuniculi* (ECLD), *E. hellem* (CDC:V257) and *E. intestinalis* (CDC:V297) were harvested from culture supernatants and processed as described (Visvesvara et al. 1991). The IIF test was performed with specific rabbit antisera on smears from faecal and urine sediment samples. Also, IIF was performed on control faeces known to contain *E. bieneusi*.

DNA extraction, purification and PCR amplification - DNA was extracted from unpreserved faecal samples by bead disruption of spores, followed by digestion with proteinase K, as previously described (da Silva et al. 1999). PCR inhibitors were removed using the QIAquick PCR Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions.

Microsporidial SSU-rRNA coding regions were amplified using the following species-specific primers: EBIEF1/EBIER1 for *E. bieneusi* (da Silva et al. 1996), SINTF/SINTR for *E. intestinalis* (da Silva et al. 1997), ECUNF/ECUNR for *E. cuniculi* and EHELF/EHELR for *E. hellem* (Visvesvara et al. 1994). A GenAmp kit (Perkin Elmer Cetus, Norwalk, CT, USA) was used for PCR amplification according to manufacturer's directions. Concentration of each primer was 0.1 µg per 50 µl final PCR reaction volume containing 1 or 0.1 µl of the purified stool sample extract. The positive controls used included 0.003 ng per 50 µl PCR reaction of the corresponding cloned SSU-rRNA coding region. Conditions for PCR reactions were: denaturing at 94ºC for 30 sec in all cases, annealing at 45ºC for 30 sec for *E. intestinalis* primers and at 55ºC for 30 sec for the rest of primers, and extension at 72ºC for 90 sec. In all cases, 35 cycles were completed. Amplification products were analysed after electrophoresis in a 2% agarose gel and visualized by staining with ethidium bromide.

Test for PCR inhibitors in purified samples - To detect samples with low DNA yield and to control the inhibitory effect in the PCR reaction**,** purified samples were spiked with 0.003 ng of the corresponding cloned SSU-rRNA. Amplification of a band of the correct size indicated the removal of PCR inhibitors in the DNA purification process.

RESULTS

Light microscopy - With Weber's chromotrope-based stain, 10 samples showed a low number of spores that stained pinkish red with the Weber's stain and measured approximately 1-1.5 µm in length in some cases and about 2.5 µm in others. Spores observed in two rabbit and in one donkey samples were larger and morphologically distinct from spores found in the rest of animal stools. Clusters of microsporidia-like spores within a vacuole inside epithelial cells were also detected in the faecal smears of one dog and one goat. A low parasite burden determined by light microscopy was detected in all cases, this requiring the examination of several slides per sample.

In 10 (45.4%) rabbits samples, *Eimeria* spp. was identified, coinciding with two microsporidia positive-samples. *Trichuris* sp. ova were detected in two dog samples that had been found positive to microsporidia. *Trichostrongylus* sp. ova were identified in one microsporidia-positive goat sample.

Parasites found in the animal faecal samples are summarized in the Table. Enteric parasites were identified in most of animals studied and polyparasitism was a generalized characteristic in all samples. Nematodes predominated over Protozoa, and larvae and/or ova in different developmental stages were frequent in herbivores (donkeys, horses, goats and cows).

IIF - Microsporidia spores present in the smears from the two microsporidia-positive rabbits reacted with the polyclonal anti-*E. cuniculi* serum in IIF. The few fluorescent spores found appeared isolated and therefore difficult to diagnose.

PCR amplification - PCR was performed on unfixed faecal samples from the microsporidia-positive animals. The DNA isolated from the five faecal samples from two dogs and one goat was amplified with *E. bieneusi* specific primers and showed a diagnostic band of 607 bp in the agarose gels (Figure). No amplification was found in any of the five samples when species-specific primers for *E. intestinalis*, *E. hellem*, and *E. cuniculi* were used.

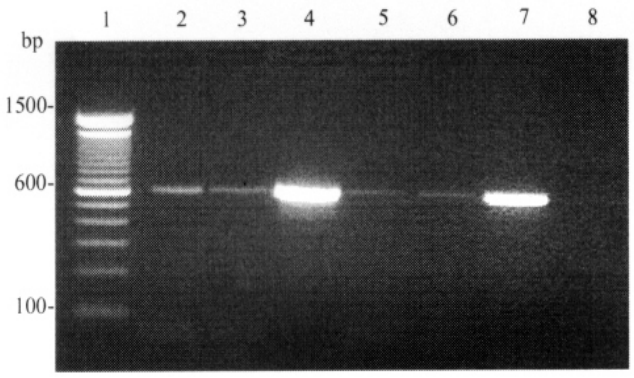
The donkey faecal samples that showed *Encephalitozoon*-like spores did not react with any of the primers used. To detect the possible PCR inhibitors in donkey faeces, we tested samples using higher dilutions of template (da Silva et al. 1997) although no amplification was observed.

TABLE

Enteroparasites detected in faeces from domestic animals

Animals (N = 75)	Enteroparasites	F %
Rabbits (N = 22)	*Encephalitozoon cuniculi*	9
	Eimeria spp.	45.4
Dogs (N = 17)	*Enterocytozoon bieneusi*	11.7
	Toxocara canis (ova)	17.6
	Trichuris sp. (ova)	11.7
	Diphylidium caninum (ova)	5.8
Cats (N =10)	*Isospora* sp.	30
	Toxocara sp. (ova)	50
Pigs (N = 4)	*Ballantidium coli* (cysts)	50
	Isospora suis	25
	Ascaris suum (ova)	50
Donkeys (N = 2)	*Strongyloides westeri* (ova and larvae)	50
Horses (N = 10)	*Trichostrongylus* sp. (ova)	50
	Strongyloides westeri (ova and larvae)	20
	Strongylus sp. (ova and larvae)	70
Goats (N = 7)	*Enterocytozoon bieneusi*	14.2
	Eimeria sp.	28.5
	Trichostrongylus sp. (ova)	57.1
Cows (N = 3)	*Nematodirus* sp. (ova)	33.3

N: number of animals examined; F: frequency of appearance



Agarose gel analysis of PCR-amplified products assayed with *Enterocytozoon bieneusi* specific primers with DNA extracted from faeces from two dogs. Lanes: 2-4: PCR with DNA from dog 1; 5-7: PCR with DNA from dog 2; 1: 100-bp ladder standard; 2, 5: 0.1 µl of sample extracts; 3, 6: 1 µl of sample extracts; 4, 7: 1 µl of sample extracts, including cloned *E. bieneusi* SSU-rRNA-coding region used as a positive control; 8: negative control

DISCUSSION

The sources of microsporidia human infections and modes of transmission, especially for *E. bieneusi*, remain uncertain. Persons or animals infected with microsporidia release spores into the environment via faecal, urine, and respiratory excretions, which all could be possible sources of infection (Bornay et al. 1998). Although, the microsporidia have been documented in one waterborne outbreak, the role of animals as the cause of contamination was not elucidated. In food, surface contamination is associated with the faecal-oral pathogens, and some data are available to indicate that animal wastes constitute a major source of contamination (Slifko et al. 2000).

Epidemiologic data are limited, and there are scarce data on animals as potential reservoir hosts for microsporidia that infect humans (Deplazes et al. 1996, Black et al. 1997, Mansfield et al. 1997, Bornay-Llinares et al. 1998, Didier et al. 1998).

In Spain, del Aguila et al. (1999) were the first to report the detection of *E. bieneusi* in faecal samples from wild and domestic rabbits and in domestic dogs, suggesting the possible zoonotic role of *E. bieneusi*, to date considered exclusively a human parasite.

E. bieneusi was identified by PCR in pig faecal samples with a prevalence of 35% (Breitenmoser et al. 1999) and, although it seems to be a common parasite in swine, no genotypes that have been identified in humans were found. However, a recent study (Rinder et al. 2000) indicate a close relationship between *E. bieneusi* strains from humans and pigs, suggesting the absence of transmission barrier between pigs and humans for this parasite.

In the present study, using Weber's chromotrope stain, IFI and PCR techniques, we detected microsporidian spores in 10 faecal samples from domestic animals (2 rabbits, 2 dogs, 1 goat, and 1 donkey) from rural villages in Galicia. Microcopic examination of spores inside the epithelial cells of faecal samples from the animals studied (dog and goat) suggests that these animals could be hosts of *E. bieneusi.*

However, we do not know whether the presence of *E. bieneusi* in the faecal samples are the result of an active infection or a simple passing of the spores through the digestive tract (del Aguila et al. 1999). Nevertheless, these results demonstrate that these animals harboured *E. bieneusi* spores and shed them into the environment. This finding will be useful to establish other possible transmission routes and to broaden the knowledge of the human epidemiology of this infection.

To date, only *E. cuniculi* infection is considered a zoonosis (Deplazes et al. 1996, Didier et al. 1996). There are three different strains of *E. cuniculi* identified by Western blot analysis of spore antigens and by random amplification of polymorphic DNA, as well as by determination of differences in the rDNA intergenic spacer region: strain type I includes rabbit isolates, strain type II includes murine isolates and strain type III, dog isolates (Didier et al. 1995). The canine and rabbit strains have been identified in three and six patients, respectively (Deplazes et al. 1996, Didier et al. 1996, Mathis et al. 1997, Weber et al. 1997).

The above results show that in two of the rabbits *E. cuniculi* spores were identified by IFI. It is unknown whether the presence of the *E. cuniculi* spores released in the faeces of these animals might signify colonization or infection by the parasite, or simply passage through the digestive tract. This could also be a disseminated infection and thus the spores appeared in the faeces by urine contamination.

In the faeces of a donkey, we found spores with a size compatible with the microsporidia of the genus *Encephalitozoon*, but these had no amplification by PCR of the DNA studied. The application of the PCR technique in the analysis of faecal samples should be performed with caution to avoid false negatives. If the parasite-DNA concentration is low, the amplification requires large volumes of sample in the reaction. Nevertheless, the presence of certain samples of PCR inhibitors allows adequate amplification only when these are more diluted (da Silva et al. 1997, Ommbruck et al. 1997). In this case, despite the repeated reactions with different DNA dilutions, amplification does not occur with the species-specific primers assayed.

Furthermore, the high degree of parasitism found should be emphasized, these being multiple infections mostly by nematode parasites. Microsporidians are intracellular parasites ranked among the protists, which means that they are eukaryotic and unicellular. They exhibit a number of important, unique features which are the basis for the systematic ranking of microsporidia, which are knowledged to constitute a phylum of their own (Wittner 1999). In particular, in the area where this study was made, helminthosis predominates over protozoosis, precisely the opposite of the situation found in other surveys in Spain, and consistent with the research performed on the presence of intestinal parasites in diverse human populations and in other mammals in Galicia (Arias 1980). The area studied (NW, Spain), with a sub-humid Mediterranean weather-type with an Atlantic tendency (Carballeira et al. 1983), includes an environment favourable for the development of infective stages of different nematode species.

The presence of microsporidia in the natural infections of animals are important for the study of clinically significant disease in humans (Rabeneck et al. 1995). With the recent improvements in diagnostic methods for detecting microsporidia, reports are being published about this infection, and as more is learned about the epidemiology, immunology, and pathology of microsporidiosis, advances in the prevention and control of microsporidiosis are more likely in susceptible mammalian hosts (Didier et al. 2000).

The detection of new *Enterocytozoon* genotypes in faecal samples of domestic animals, together with recent reports of detection of *E. bieneusi* in environmental samples, suggests that microsporidia of the genus *Enterocytozoon* is ubiquitous and has many genotypes in various infected animal species (Mathis et al. 1999).

In zoonotic studies, it is important to investigate all the possible reservoirs for each causal pathogenic agent in any geographical area (Lores et al. 1994) and thus it is crucial to confirm the zoonotic role of *E. bieneusi*, as this fact may change the established idea of *E. bieneusi* as a human parasite and possibly characterize it as a zoonotic parasite. As microsporidia are released into the environment via stool, urine and respiratory excretion, possible sources of infection may be persons or animals infected with this group of parasites (del Aguila et al. 1999).

Finally, the role of animals in the transmission of microsporidial human infections requires further study. Although animals have been implicated in some opportunistic infections among HIV-infected persons, the overall risk of transmission from contact with domestic animals is unknown, but may be further reduced. Nevertheless immunodeficient patients, especially with Aids, should be informed with practical suggestions designed to reduce this low risk.

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