Encephalitozoon intestinalis (Septata intestinalis) is the second most prevalent microsporidian species infecting humans, but it has not been described in other animal species. This investigation examined 10 domestic animal stool samples (8 mammalian, 2 avian) containing spores detected by anti-Encephalitozoon monoclonal antibody immunofluorescence (FA). The presence of E. intestinalis but not Encephalitozoon hellem or Encephalitozoon cuniculi was confirmed in 6 of 8 mammalian stool samples by species-specific FA and polymerase chain reaction. Clusters of spores inside epithelial cells were observed in feces of five mammals (donkey, dog, pig, cow, and goat) using "quick-hot" Gram-chromotrope stain. None of the 10 samples reacted with anti-E. hellem or anti-E. cuniculi sera, nor were they amplified with species-specific primers for E. hellem and E. cuniculi. To our knowledge, this is the first identification of E. intestinalis in animals other than humans. The data shown herein suggest the possibility that E. intestinalis infection may be zoonotic in origin.

Microsporidia are spore-forming, amitochondrial protozoa grouped in the phylum Microspora. Nearly 1000 species belonging to >100 genera are included in this phylum [1]. Microsporidia infect hosts belonging to many invertebrate phyla and all five classes of vertebrates, including primates [2]. Nevertheless, only seven genera (Encephalitozoon [Septata], Enterocytozoon, Nosema, Pleistophora, Trachipleistophora, and Vittaforma) as well as unclassified isolates named Microsporidium species, are known to cause disease in humans. The taxonomic position of Septata intestinalis [3] is not well established, as it was recently proposed to be merged into the Encephalitozoon genus and to be renamed as Encephalitozoon intestinalis [4, 5].

Microsporidia have been increasingly recognized as opportunistic pathogens of immunodeficient patients [6]. Among the microsporidian species that infect humans, Enterocytozoon bieneusi is the most prevalent, causing diarrhea and pulmonary and hepatobiliary disease [6]. E. intestinalis is the second most prevalent microsporidian, causing gastrointestinal infections, both local and disseminated [7–9].

Intracellular development of E. intestinalis within a septated parasitophorous vacuole in tissue samples or in vitro culture is morphologically distinct from that of Encephalitozoon hellem and Encephalitozoon cuniculi [3, 5, 10, 11]. However, spores of these three microsporidia are difficult to distinguish, even by electron microscopy, biochemical, immunologic, or molecular methods are needed for species identification [3, 5, 10–12].

The microsporidian E. cuniculi causes diseases in ~30 reported mammalian hosts, including rabbits, rats, mice, muskrats, horses, dogs, arctic foxes, domestic cats, leopards, baboons, and humans [2]. E. cuniculi is also the only microsporidian species infecting humans that has been isolated from a variety of mammals [2, 12, 13]. Additionally, reports of disease due to Encephalitozoon species in birds have been published, but identification at the species level was not performed [2]. However, E. hellem was identified unequivocally in a budgerigar (Melopsittacus undulatus). The parasite was found in the enterocytes, lamina propria, and liver of budgerigar chicks [14]. Recently, E. bieneusi was identified for the first time in pigs [13] and in simian immunodeficiency virus–inoculated monkeys (Macaca mulata, Macaca cyclopis, and Macaca nemestrina) [15]. E. intestinalis, however, has not been re-
ported in any animal species other than humans nor detected in environmental samples.

In this study, we used microscopic, immunologic, and molecular methods to demonstrate the presence of *E. intestinalis* spores in stools of mammals other than humans. This could be an indication that animals serve as reservoirs of this parasite in nature.

**Materials and Methods**

**Animal stool samples.** Domestic animal stool samples (n = 271; 172 mammalian and 99 avian species) were collected during a cross-sectional study conducted during the summer and fall of 1996 in two rural communities (San Jose el Rincón [J] and Libertad Tecola [L]) located in central Mexico 30 km from Puebla City [16]. Of these animals, 19 (11.0%) of 172 mammalian and 16 (16.2%) of 99 avian species had fecal spores detected by monoclonal antibody (MAb) 3B6-based IFA. Samples from each village were collected at the same time. Ten samples were selected for this study, including: LD002-Ch1 (goat), LD002-G1 (kitten), LD006-J1 (turkey), JB007-C7 (piglet), J1022-Y1 (chicken), LA023-V3 (cow), JB003-G (cat), JB003-R (dog), JB003-B1 (donkey), and LD010-C2 (pig). These samples contained spores that were detected by IFA with MAB 3B6, which binds to the exospore of *Encephalitozoon* species [17]. Samples were stored in 10% formalin for several months. At the time of this study, samples were concentrated by ethyl acetate, resuspended in 2.5% potassium dichromate, and stored at 4°C until used.

**Microscopy.** The samples were stained with the “quick-hot” Gram-chromotrope stain [18] for optical microscopy analysis. For transmission electron microscopic studies, stools were first washed three times with PBS and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 3 h at 4°C. The samples were dehydrated in ethanol-propylene oxide solution and embedded in Spurr medium. Ultrathin sections were cut and stained with Reynold’s lead citrate (10 min) and with 2% (wt/vol) uranyl acetate (10 min) in distilled water. Sections were examined with a Zeiss EM-900 transmission electron microscope.

**IFA.** Stool samples were washed and concentrated using ethyl acetate sedimentation. Concentrates were used to detect *Encephalitozoon* species by IFA using the anti-*Encephalitozoon* species MAb 3B6 [17]. Briefly, stool samples were mounted on poly-L-lysine-coated 10-well slides, blocked with bovine serum albumin, and incubated sequentially with anti-*Encephalitozoon* species MAb 3B6 (ascites, 1:5000 dilution) and with fluorescein isothiocyanate–labeled goat anti–mouse IgG (1:100 dilution; Kirkegaard & Perry, Gaithersburg, MD).

Polyclonal antisera prepared in rabbits against different microsporidial isolates from patients with AIDS, including *E. hellem* (CDC: 0291-V213) isolated from urine [19], *E. cuniculi* (CDC: V282) isolated from urine and sputum [10], and *E. intestinalis* (CDC: V297) isolated from urine [11], were also used to identify the species involved. Briefly, serial dilutions of the antisera (beginning at 1:100) were prepared and tested for ascertaining the optimal reactivity in the IFA with the homologous antigen but with no cross-reactivity with heterologous antigen. The optimal dilution was found to be 1:400. IFA was performed as previously described [19]. Briefly, 5 µL of each stool sample was directly applied to 4 individual wells in multwell slides, and each sample was probed with a 1:400 dilution of anti–*E. hellem*, anti–*E. cuniculi*, or anti–*E. intestinalis* sera and incubated (37°C) for 30 min. The slides were washed three times with PBS and then incubated with fluorescein isothiocyanate–labeled goat anti–rabbit IgG (1:800 dilution; Cappel Laboratories, Westchester, PA). Slides were washed and mounted with a buffered solution containing an antifouquer. Spores were visualized with an Olympus BH epifluorescence microscope at >400 magnification.

**Polymerase chain reaction amplification (PCR).** DNA was extracted from animal stool samples by bead disruption of spores, followed by digestion with protease K, as previously described [20]; PCR inhibitors were removed using the QIAquick PCR kit (Qiagen, Chatsworth, CA) according to the manufacturer’s instructions.

Identification of microsporida at the species level was performed using diagnostic primers for *E. intestinalis*, named SINTF/SINTR, which specifically amplify a 520-bp diagnostic fragment from the *E. intestinalis* small subunit of the rRNA (SSU-rRNA) coding region [21]. In order to detect samples with low DNA yield and control the inhibitory effect in the PCR reaction, volumes of 10, 1, and 0.1 µL of each stool sample extracts were used as templates. In addition, the presence of PCR inhibitors in the stool extracts was assessed by spiking each sample with 0.1 ng of *E. intestinalis*–cloned SSU-rRNA coding region. Products of amplification were resolved on a 2% agarose gel (SeaKem GTG; FMC BioProducts, Rockland, MA) and visualized by staining with ethidium bromide. In addition, all samples were tested with species-specific primers for *E. hellem* (EHELR/EHELF) and *E. cuniculi* (ECUNR/ECUNF) [19].

**Results**

**Light microscopy.** Using the quick-hot Gram-chromotrope stain, all 10 samples showed microsporidian spores of ~1.5–2.0 µm in length (figure 1A). Spores found in avian stools were larger and morphologically distinct from spores found in mammalian stools. The morphologic difference between spores was initially observed in 1 avian species (chicken, figure 1F) and 1 mammal (donkey; figure 1G) stool sample stained by IFA using the MAB 3B6. Clusters of *Encephalitozoon*-like spores within a parasitophorous vacuole inside epithelial cells were also seen in the fecal smears of 5 different animals (donkey, dog, pig, cow, and goat) (figure 1B–E).

**Electron microscopy.** *Encephalitozoon*-like spores were identified by electron microscopy in stool samples from the 3 animals (cow, pig, and goat) that had the highest parasite burden, as determined by light microscopy. Examination of goat stool revealed the presence of degenerating but recognizable host cells containing both developing and mature microsporidia. In one of these cells (possibly a sloughed enterocyte), a clearly recognizable parasitophorous vacuole containing immature and mature microsporidian spores was present within the cytoplasm (figure 2A). The spores contained an average of six to seven turns of the coiled polar tube arranged in a single
layer, and the size and ultrastructural features were consistent with members of the genus *Encephalitozoon* (figure 2E). In cow stool, numerous extracellular mature microsporidian spores were present with morphologic features similar to those in goat stool (figure 2D). In addition, hollow, or empty, microsporidian spores were identified, as were extruded microsporidian polar tubules (figure 2C), indicating that the spores had discharged their contents in the feces. In stool examination of the pig, only empty microsporidian spores were identified (figure 2B).

**IFA.** Spores that reacted with the anti-*Encephalitozoon* MAb 3B6 were observed in all samples. However, spores in 6 of the 8 mammalian stool samples reacted brightly with the rabbit anti-*E. intestinalis* serum. Bright fluorescence indicated a specific reaction of anti-*E. intestinalis* serum with spores in the smears. Both avian samples were negative when tested with anti-*E. intestinalis* serum. None of the 10 samples reacted with rabbit anti-*E. hellem* or rabbit anti-*E. cuniculi* sera. Spores were present with morphologic features similar to those in goat stool (figure 2D). In addition, hollow, or empty, microsporidian spores were identified, as were extruded microsporidian polar tubules (figure 2C), indicating that the spores had discharged their contents in the feces. In stool examination of the pig, only empty microsporidian spores were identified (figure 2B).

**PCR amplification.** The *E. intestinalis* diagnostic DNA fragment of 520 bp was amplified with the SINTF/SINTR PCR primer pair from all 6 mammalian stool samples that reacted positively by IFA with the *E. intestinalis* specific anti-serum (figure 2B). For the goat sample (lanes 1–4), no amplification was seen when 0.1 μL of the template was used (lane 3), whereas *E. intestinalis*—
Figure 2. Transmission electron microscopy of microsporidia in stool specimens from goat, pig, and cow. A, Goat stool showing degenerated cell with preserved nucleus (nuc), including nucleolus and nuclear membrane and surrounding cytoplasmic debris (Cy). Intracytoplasmic parasitophorous vacuole (arrowheads) is still recognizable and contains *Encephalitozoon*-type mature spores (M) and immature sporont (Sp) and sporoblast (Sb). No septations are identified between spores, but because this represents necrotic cell shed into feces, it is possible that these distinctive structures have degenerated. Animal Ch1, original magnification, ×19,200. B, Pig stool showing empty microsporidian spores (ES) and cross-section of polar tubule (arrow; animal C2, original magnification, ×38,000). C, Extruded microsporidian polar tubules (Pt) in cow stool. Fine structure of polar tubule is well visualized in cross-sections (arrows; animal V3, original magnification, ×38,000). D, High magnification of mature microsporidian spore in cow stool (animal V3, original magnification, ×112,000). E, Cross-section of mature microsporidian spore from goat stool. Present are electron-dense exospore (exo), electron-lucent endospore (endo), and 7 coils (1–7) of polar tubule (animal Ch1, original magnification, ×112,000).
Figure 3. A. Agarose gel analysis of polymerase chain reaction (PCR)–amplified products using species-specific primers for Encephalitozoon intestinalis. Lanes 1–6: animal stool samples. Lane 1, goat (1 μL); lane 2, piglet (1 μL); lane 3, cow (1 μL); lane 4, dog (10 μL); lane 5, donkey (1 μL); lane 6, pig (0.1 μL); lane 7, negative control; lane 8, positive control. Lane S contains 100-bp ladder standard. Numbers to right of gel are DNA fragment sizes (in base pairs). B. Agarose gel analysis showing PCR-amplified products using different concentrations of purified DNA from animal stool samples. Lanes 1–4: goat stool, lanes 5–8: donkey stool; lanes 9–12: chicken stool; lanes 1, 5, and 9: 10-μL sample volume; lanes 2, 6, and 10: 1-μL sample volume; lanes 3, 7, and 11: 0.1-μL sample volume; lanes 4, 8, and 12: spike control (10 μL of respective purified sample plus 10 μL of DNA preparation containing 0.1 ng of Encephalitozoon intestinalis—cloned small subunit of rRNA). Lane S contains the 100-bp ladder standard. Nos. at right of gel are standard DNA fragment sizes (in base pairs).

Discussion

The sources of microsporidia infecting humans and modes of transmission, particularly for E. intestinalis, remain uncertain. Persons or animals infected with microsporidia release spores into the environment via stool, urine, and respiratory excretions, which all could be possible sources of infection. Epidemiologic data are limited, and there are scarce data on animals as potential reservoir hosts for microsporidia that infect humans (i.e., E. bieneusi, E. intestinalis, E. hellem, and E. cuniculi) [12–15].

E. cuniculi infection is actually considered a zoonosis [12, 13]. There are 3 different strains of E. cuniculi (i.e., rabbit, mouse, and dog) 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. The canine and rabbit strains have been identified in 3 and 6 patients, respectively [12, 13, 22]. On the other hand, there is only one report of E. hellem infection in birds [14]. Definitive species identification in this case was achieved by Southern blot analysis using an oligonucleotide sequence specific for E. hellem.

In our study, we demonstrated, with both quick-hot Gram-chromotrope staining and immunofluorescence reactivity with MAb 3B6, the presence of microsporidian spores in 10 stool samples from domestic animals from 2 villages in central Mexico. However, although the MAb 3B6 recognized spores seen
in the 2 avian hosts, they were larger than those seen in the mammalian hosts. This is not surprising because MAb 3B6 also recognizes insect microsporidia [17]. Similarly, microsporidia spores seen by IFA using MAb 3B6 and after the quick-hot Gram-chromotrope staining in the stool samples from the cat and the kitten were negative when analyzed either by PCR with *E. intestinalis*, *E. hellem*, and *E. cuniculi* diagnostic primers or IFA using anti-*E. intestinalis* serum. This suggests that these spores do not belong to microsporidia of the genus *Encephalitozoon*. Spores of *Encephalitozoon* species in feces were identified in 7.84% (20/255) of persons living in these 2 communities [16]. Ultrastructure typical for *Encephalitozoon* species was observed by electron microscopy. In an attempt to identify the microsporidian species, species-specific techniques (IFA and PCR) for identification of *E. hellem*, *E. cuniculi*, and *E. intestinalis* were used. In 6 hosts (2 pigs, 1 dog, 1 cow, 1 goat, and 1 donkey), *E. intestinalis* spores were identified using polyclonal rabbit anti-*E. intestinalis* serum and subsequently confirmed by DNA amplification using a species-specific pair of primers [21]. The presence of spores in the stools of these animals could mean colonization by the parasite or simply passing of the spores through their guts. However, the presence of spores inside the epithelial cells found in stools of all mammalian species studied suggests that these animals were probably infected with the microsporidia and may serve as hosts for this microsporidian species. To our knowledge, this is the first report that specifically identifies *E. intestinalis* in animals other than humans. On the basis of these results, we suggest that these animals harbored *E. intestinalis* spores and shed them into the environment. Nevertheless, whether these animals are the source of human infection, either by the oral route (contaminated food or water) or by the aerosol route, remains to be demonstrated. Oral transmission of microsporidia has been documented in experimental studies using animals [2, 23]. In addition, two previous reports of *E. cuniculi* infection in AIDS patients suggested that *E. cuniculi* was acquired by the oral route [22, 24]. The possibility of aerosol transmission has also been discussed by Schwartz et al. [25] after they performed the first complete autopsy of a patient with AIDS and systemic microsporidiosis due to *E. hellem*. Further studies are necessary to identify whether *E. intestinalis* also exhibits the same pattern of transmission.

It is important to emphasize that application of PCR for analysis of stool samples has to be conducted with caution to avoid misinterpretation of false-negative results. When the yield of the parasite DNA is low, amplification is observed only when samples are tested at higher amount of template in the PCR mixture. Conversely, in samples that have PCR inhibitors, amplification is observed only at a higher dilution of template [20]. In our study, we tested all samples using three different template volumes and spiked them with positive controls in separate reactions to guarantee proper interpretation of the results. Our criteria to consider a true negative sample implied that no amplification should occur in any of the dilutions, with no visible inhibition of the spike control.

Among microsporidian species that infect immunocompromised persons, *E. intestinalis* is certainly the second most prevalent after *E. bieneusi* [7, 9]. Additionally, seroprevalence studies have demonstrated that *Encephalitozoon* species, including *E. intestinalis*, may also be common among immunocompetent subjects from tropical and temperate regions [26, 27]. *E. intestinalis* can disseminate, causing infections in different organs and tissues of immunodeficient human hosts. The evidence herein that *E. intestinalis* can be found in animals other than humans serves as a foundation for further investigation to understand the distribution of *E. intestinalis* in different animal species. In addition, it will be important to define other possible excretion routes of spores into the environment.

Of the domestic animals studied, only mammals shed *E. intestinalis* in their feces. Since we found *E. intestinalis* in domestic animals (dog and donkey) as well as in animals that are used for human consumption (cow, goat, and pig), it will be important to discover whether infections by *E. intestinalis* are disseminated in these animals and which organs are the main target of infection. In vitro cultivation of animal *E. intestinalis* strains will be of invaluable importance to define new molecular markers to extend our epidemiologic studies. The population at risk will benefit from such investigations, and new prevention measures can be implemented if *E. intestinalis* infection is proven to be a zoonosis.

Acknowledgments

The excellent technical assistance of Patricia Cruz, Brian Curran, and numerous University of Puebla students is greatly appreciated. The cooperation of local authorities in the towns of San Jose el Rincón and Libertad Tecola, Puebla, Mexico, is greatly appreciated. We thank Carol Bender for her outstanding assistance in coordinating all efforts related to international work. We are indebted to Meridian Diagnostics, Cincinnati, for the kind donation of reagents to facilitate the execution of this work.

References


