Identification of *Encephalitozoon* and *Enterocytozoon* (Microsporidia) Spores in Stool and Urine Samples Obtained from Free-Living South American Coatis (*Nasua nasua*)

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This study emphasizes the importance of free-living coatis as a potential source of microsporidian infection for humans living in large cities. We found 19 (31.7%) positive results among 60 fecal samples analyzed by PCR-based analysis and the Gram–Chromotrope staining technique (11.7% were positive for *Encephalitozoon cuniculi*, 6.7% for *E. intestinalis*, 6.7% for *E. hellem*, and 6.7% for *Enterocytozoon bieneusi*). Only 5 (8.4%) urine samples tested positive for *E. cuniculi* as assessed by the two techniques.

Microsporida are single-celled, intracellular, eukaryotic parasites. These obligate intracellular parasites, which have recently been reclassified from protozoa to fungi (17), affect a wide variety of vertebrate and invertebrate hosts of all animal phyla (2). Microsporidia, especially *Encephalitozoon* spp. and *Enterocytozoon* spp., are considered to be emerging pathogens of increasing importance and a cause of opportunistic infection in immunosuppressed patients (11). *Encephalitozoon intestinalis* causes diarrhoea and chronic wasting, while *E. cuniculi* commonly affects the central nervous system, the respiratory system, and the digestive system (14, 18). *Encephalitozoon hellem* was first described as a new species in 1991 after being identified as the cause of disease in three AIDS patients (4) and has since been documented as a cause of various clinical syndromes, including respiratory, urogenital, and disseminated diseases, that often affect immunosuppressed patients (13, 15). *Enterocytozoon bieneusi* is the most prevalent species identified in humans and primarily contributes to diarrhoea, especially in AIDS patients (11).

The epidemiology of human and animal microsporidiosis has been the focus of many studies, as the prevalence of the disease has increased greatly in recent years (9, 14). Both infected people and animals are sources of infection and contaminate the environment by releasing spores in feces, urine, and other secretions (10). Spores of microsporidia have been detected in both food and water (7). Identifying the possible source of infection for humans and other animals is a fundamental step to understanding zoonotic transmission. To contribute to the knowledge of the epidemiology of microsporidiosis with a focus on the zoonotic potential, we used a combination of PCR and staining to determine the occurrence of *E. cuniculi*, *E. hellem*, *E. intestinalis*, and *E. bieneusi* in coatis (*Nasua nasua*). We believe that these studies will be important in understanding the epizootiology of human infections.

For the investigation of microsporidian spores, a total of 60 stool and urine samples obtained from South American coatis (*Nasua nasua*) were examined. These animals were captured in the Tietê Ecological Park, São Paulo State, for surgical neutering to control this local population of the species. To avoid any risk of transmitting diseases or accidents in the handling of animals, all procedures were performed under the supervision of a veterinarian.

Thirty-five of the animals were female, and 25 were male; all animals analyzed were adults and were in good health. Fecal samples obtained from the rectum of each animal were kept for a maximum of 24 h in tubes with a solution of 10% buffered formalin (pH 7.2 to 7.4). From the preserved and homogenized samples, 10–μl amounts of feces were collected to generate thin films. After fixation with methanol, slides were submitted for Gram–Chromotrope analysis (12).

Urine samples were collected during surgery by puncture of the bladder with a syringe and needle and subsequently stored at 4°C until they were analyzed. Urine samples were centrifuged at 8,000 × g for 5 min, the supernatant was discarded, and the pellet was used to make thin-smear slides, which were then fixed with methanol and submitted for Gram–Chromotrope analysis. Parasitic forms were measured with an ocular micrometer (using ×400 and ×1,000 magnification). Photographic documentation was performed using a Zeiss Axioshop 2 optical photomicroscope with a Pixera digital camera (Pixera Corporation, Santa Clara, CA) coupled to a 700-MHz Pentium computer. Measurements of spores were recorded in micrometers with Sigma Scan Pro 5 software (Jandel Scientific Corporation, Corte Madera, CA).

For DNA isolation, 200 μl of either formalin-fixed fecal or urine sediment samples were used. For the fecal samples, formalin was removed by washing and centrifuging samples with saline, followed by heating the samples for 10 min at 100°C. After sodium dodecyl sulfate–proteinase K treatment (2 h at 55°C), DNA was isolated with QIAamp tissue kit spin columns (QIAGen, Hilden, Germany). Microsporidian samples used as controls included tissue culture-harvested *Encephalitozoon cuniculi*, *Encephalitozoon hellem*, and *Encephalitozoon intestinalis*, and for *Enterocytozoon bieneusi*, fecal specimens from humans were used. For identification of species, extracted DNA was assessed by PCR using PMP1/PMP2 primers for *E. hellem* (5), ECUNF/ECUNR primers for *E. cuniculi* (6), SINTF/SINTR primers for *E. intestinalis* (3), and EBIEIF/EBIER1 primers for *E. bieneusi* (3). The thermocycling conditions were applied as follows: 95°C for 5 min and 35 cycles of
TABLE 1 Coatis positive for microsporidian infection by either light microscopy or PCR

<table>
<thead>
<tr>
<th>Species</th>
<th>Light microscopy</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Feces</td>
<td>Urine</td>
</tr>
<tr>
<td><em>E. cuniculi</em></td>
<td>7 (11.7)</td>
<td>5 (8.4)</td>
</tr>
<tr>
<td><em>E. hellem</em></td>
<td>4 (6.7)</td>
<td></td>
</tr>
<tr>
<td><em>E. intestinalis</em></td>
<td>4 (6.7)</td>
<td></td>
</tr>
<tr>
<td><em>E. bieneusi</em></td>
<td>4 (6.7)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>19 (31.7)</td>
<td>5 (8.4)</td>
</tr>
</tbody>
</table>

94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a 10-min incubation at 72°C. Amplicons were analyzed via electrophoresis in 1% agarose and viewed after staining with ethidium bromide.

We identified 24 (40%) animals that tested positive for the presence of microsporidian species in urine and/or fecal samples (Table 1). All affirmative cases tested positive for both techniques utilized (i.e., staining and PCR). Samples showed various numbers of purple-stained spores with the characteristics of microsporidia as manifested via Gram-Chromotrope staining. Using light microscopy, the microsporidian spores appeared as oval structures, with some measuring 2.2 μm to 3.2 μm in length by 1.0 μm to 1.6 μm in width.

Based on fecal sample examination, single-species infections in coatis were detected as follows: 7 (11.7%) coatis tested positive for *E. cuniculi*, 4 (6.7%) coatis were positive for *E. intestinalis*, 4 (6.7%) animals tested positive for *E. hellem*, and 4 (6.7%) animals were positive for *E. bieneusi*, which accounts for 19 (31.7%) of the positive cases (Table 1). No fecal sample presented with more than one type of microsporidian species.

Five (8.4%) urine samples were found positive for *E. cuniculi* according to the two techniques. Only spores of *E. cuniculi* were detected, which was expected as this species is responsible for kidney infections in many mammals. None of the animals tested were positive at the same time in the examination for feces and urine. Unfortunately, it was not possible to collect more than one fecal or urine specimen to avoid false-negative results, as the animals were released after recovery from surgery. The association between infection and clinical manifestations was not assessed, as these animals were captured exclusively for the surgical procedure and were later released into the park.

Disease emergence or reemergence is often a consequence of social and technological changes and frequently manifests in an unpredictable manner. It has been estimated that 75% of emerging diseases display zoonotic characteristics (1, 11). Many factors influence the emergence of zoonoses, such as environmental change and land use, changes in demographics, changes in technology and industry, increasing international travel and commerce, the breakdown of public health measures, and microbial adaptation (1).

As in many other cities of the world, greater São Paulo occupies a large area that includes highly urbanized regions, forested areas, and natural parks. On weekends, when a large population visits these natural areas, people sometimes come into contact with wild animals or the environment that may be contaminated by pathogens eliminated by infected animals. In addition, many wild animals approach people’s homes in search of food and eliminate excreta locally, thereby contaminating the urban area.

We have demonstrated that free-living South American coatis can be a source of infection of the genera *Encephalitozoon* and *Enterocytozoon* affecting the human population in large cities, as 40% of the coatis investigated in this study tested positive for these parasites.

Many studies have demonstrated the presence of parasites in a wide variety of domestic and wild animals, such as rabbits, mice, rats, dogs, blue foxes, red foxes, wild dogs, feral mink, monkeys, and many species of birds and farm animals (11). However, this is the first study to demonstrate the presence of *Encephalitozoon* and *Enterocytozoon* spores in the feces and urine of coatis.

Most positive cases were observed following examination of feces, in which *E. cuniculi* was most prevalent (31.7%). *E. cuniculi* has been reported to infect a wide range of hosts, including humans, other mammals, and birds. It is likely that *E. cuniculi* is a natural pathogen in humans, and its zoonotic origin is evident (16), as described regarding two of six patients from Switzerland, all of whom were infected with *E. cuniculi* (16). Moreover, spores of microsporidia are highly resistant in the environment and can survive for several months in humid conditions (9).

The other *Encephalitozoon* species identified had a lower prevalence (6.7% for both *E. hellem* and *E. intestinalis*), although their presence in wild animals indicates an increasing number of sources of infection for humans. These studies greatly enhance the understanding of the disease’s epidemiology.

We describe the identification of *E. cuniculi* spores found in urine specimens (5/60, 8.4%) isolated from coatis. *E. cuniculi* has been described as a cause of encephalitis and nephritis in rabbits. The finding of this species in urine of South American coatis may represent a similar environmental niche for this parasite (17). Moreover, spore elimination from contaminated urine contributes to environmental pollution, especially of water (7). Previously, we have identified microsporidian spores in the stool samples of small rodents (montane akodonts, prehensile-tailed porcupines, and pigmy rice rats), marsupials (gray slender mouse opossums and big-eared opossums), and hairy-legged vampire bats in an area of deforestation for the construction of a dam (8). Further augmenting our studies, we describe here *Encephalitozoon* and *Enterocytozoon*-infected coatis in a park around a major river in the region of the Tieté River. It is important to note that many wild animals can participate in the epidemiological chain of transmission of these parasites that cause significant human diseases in immunosuppressed patients.

REFERENCES