Identification of Potentially Human-pathogenic *Enterocytozoon bieneusi* Genotypes in Various Birds

Running title: Microsporidia in birds

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Enterocytozoon bieneusi was detected in 24 of 83 samples from Columbiformes, Passeriformes and Psittaciformes. It was identical to or closely related to the Peru6 genotype, which was previously found in humans in Peru. Thus, various birds can be a significant source of environmental contamination of potentially human-pathogenic E. bieneusi.
The four most common human microsporidian species, *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *Encephalitozoon hellem* and *Encephalitozoon cuniculi*, have been reported in a wide range of domestic and wild animals (3, 7, 9, 11, 13, 14, 18). Thus, it has been debated for some time now whether animals, namely birds, could be a source of infection of microsporidiosis to humans (9, 15). This is especially true for *E. bieneusi*, the most common microsporidian parasite in humans.

Thus far, *E. bieneusi* has been reported in two of eight symptomatic chickens examined in Germany and 17 of 124 normal pigeons examined in Spain (5, 12). The zoonotic potential of *E. bieneusi* from birds is not clear. Only four *E. bieneusi* samples of bird origin have been genotyped. The two samples in chicken in Germany had *E. bieneusi* genotype J, one of the several host-adapted genotypes found in cattle (12). Two sequences from pigeons in Spain also produced two *E. bieneusi* genotypes different from any genotypes described so far (5). However, the knowledge of the broad host range of these species does not by itself present direct evidence that any of these hosts function as a reservoir for human infection. The finding by molecular tools of previously unrecognized intraspecific genetic differences has improved our understanding of the epidemiology and zoonotic transmission of these microorganisms (9).

This study intended to examine the occurrence of *E. bieneusi* in several bird species in close contact with humans (pet birds and pigeons from public parks), and to characterize the parasites found at the genotype level.

**Bird specimens.** A total of 83 birds in the Orders Psittaciformes, Passeriformes and Columbiformes, including 39 pet cage-birds and 44 pigeons, were surveyed for
possible infection with microsporidia. The Psittaciformes studied included *Agapornis* sp. (n=1), *A. fischeri* (n=5), *A. personatus* (n=3), *A. roseicollis* (n=4), *Amazona aestiva* (n=1), *Forpus coelestis* (n=1), *F. conspicilattus* (n=1), *Melopsittacus undulatus* (n=4), *Nymphicus hollandicus* (n=3), *Platycercus eximius* (n=1), and *Psittacus erithacus* (n=8). The Passeriformes included *Bathilda ruficauda* (n=1), *Erythrura gouldiae* (n=1), *Leiothrix lutea* (n=1), *Lonchura domestica* (n=2), *Padda oryzivora* (n=1), and *Serinus canaria* (n=1). They were mostly from an avian breeder (31 birds), and several pet owners (8 birds) in Lisbon, Portugal. Faecal droppings were collected from these birds. The pigeons (*Columba livia*, Order Columbiformes) studied were captured in two public parks in Lisbon by personnel from Lisbon Health Department. These birds were submitted for necropsy, when the intestinal content was collected for this study.

**E. bieneusi genotyping.** DNA was extracted from feces or intestinal content using the Mini-BeadBeater/silica method or the FastDNA SPIN kit for soil (6, 11). A nested PCR protocol was used to amplify a fragment consisting of the partial small subunit and large subunit and the entire internal transcribed spacer (ITS) region of the rRNA gene of *E. bieneusi* (18). For primary PCR, a fragment of 410 bp was amplified. For secondary PCR, a fragment of 392 bp was amplified from 2.5 µl of the primary PCR product reaction. PCR products were analyzed by 2% agarose gel electrophoresis and ethidium bromide-staining. The secondary PCR products with the expected size were sequenced in both directions on a CEQ 2000XL instrument (Beckman Coulter Inc., Fullerton, CA.) or an ABI3100 automated sequencer (Applied Biosystem, Foster City, CA.). The accuracy of the nucleotide sequence was confirmed by sequencing two
separate PCR products from the same specimen. The sequences obtained were analyzed together with the reference sequences from the GenBank database using the BLASTN (www.ncbi.nlm.nih.gov) and ClustalX (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) programs. The unique *E. bieneusi* ITS sequences obtained were deposited in GenBank under accession numbers DQ425107, and DQ425108.

Occurrence of *E. bieneusi* in birds. ITS PCR products of the expected size (~392 bp) were obtained from 24 (19 pigeons, two African grey parrots, one cockatiel, one Fischer’s lovebird and one Star finch) of the 83 birds examined (Table I). Thus, *E. bieneusi* was identified in specimens from all three orders of birds studied. However, pigeons had a significantly higher frequency (43.2% versus 12.8%) of microsporidian infection than pet birds ($X^2 = 9.27; P = 0.0023$). None of the infected birds displayed clinical signs, except for one parrot that had decreased appetite and weight loss one week before death.

Genotypes of *E. bieneusi* in birds. DNA sequencing of PCR products was successful for specimens from 21 birds. All sequences obtained belonged to *E. bieneusi*. Sequence analysis revealed that the 16 pigeons and one love bird were infected with an *E. bieneusi* genotype previously reported in AIDS patients as the Peru6 genotype (17). One pigeon and one parrot had an *E. bieneusi* genotype very similar to Peru6, but with one nucleotide change (G to A) near the 3’end of the PCR fragment. Two pigeons had a mixed Peru6 and Peru6 variant judged by the electropherograms of DNA sequencing and repeated PCR analyses.
Public health significance. In this study, *E. bieneusi* was identified by PCR in the specimens of 28.9% of the birds sampled. Most infected birds were apparently healthy, and might serve as asymptomatic carriers for microsporidian species, as already suggest for *Encephalitozoon hellem* by others (15). Previous reports of clinical microsporidiosis in birds involved mostly young animals and those co-infected with other pathogens (viruses and bacteria) (2). Thus, microsporidia in avian hosts could be largely opportunistic pathogens as seen in humans.

Previously, the majority of microsporidian cases in birds were documented in species of in the Order Psittaciformes and involved almost exclusively *Encephalitozoon hellem* (15). *Enterocytozoon bieneusi* was only seen in two chicken in the Order of Galliformes and 17 pigeons in the Order of Columbiformes (5, 12).

Airborne transmission, previously, proposed for *E. hellem* (2), also might occur for *E. bieneusi*, since this species has been found in lungs of AIDS patients (8, 16). As shown in this study, microsporidian spores are commonly shed in bird excrements. Because bird droppings dry quickly and produce dust, inhalation of dust containing viable spores into the respiratory tract, as it happens for *Histoplasma capsulatum* and *Chlamydia psittaci* (2), might initiate an infection, especially in immunocompromised persons.

A high intra-specific variability in *E. bieneusi* has been described previously based on sequence differences of the ITS of the rRNA gene. Over 50 genotypes of *Enterocytozoon* spp. have been reported, with many distinct and probably host-adapted genotypes associated with specific groups of animals, which probably have no significant public health importance (20). However, a large group of closely related *E. bieneusi*
genotypes have no strict host specificity, and are frequently found in both humans and animals (18). In the present study, the genotype found in birds had ITS sequences identical or similar to an *E. bieneusi* genotype previously reported in Peruvian AIDS patients, the Peru6 genotype (17). To our knowledge, this is the first time that an *E. bieneusi* ITS genotype identical to one found in humans has been found in a variety of birds (18 pigeons, and one lovebird). Unlike previous studies conducted in mammals, which had shown that humans and animals in a geographic area are usually infected with multiple *E. bieneusi* genotypes (1, 17, 18, 19), birds in this study were infected with only two closely related genotypes. This could be the result of the highly mobile nature of birds, which may select more transmissible genotypes.

The finding in birds of an ITS genotype identical to the one found in humans suggests a high zoonotic potential of avian *E. bieneusi*. This finding provides further support to the suggestion of a lack of transmission barriers in *E. bieneusi* between different animal species or even different taxonomic classes of the hosts. Moreover, the high prevalence of infection in pigeons and the large number of this avian species in Portugal and other European countries (5) indicate that they might be a potential source of human infection and a significant source of environmental contamination.

REFERENCES


Table I – *Enterocytozoon bieneusi* genotypes identified in different birds.

<table>
<thead>
<tr>
<th>Order</th>
<th>Common name/Scientific name</th>
<th>Specimen</th>
<th>E. bieneusi prevalence</th>
<th>E. bieneusi genotype</th>
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<td>Psittaciformes</td>
<td>Lovebird/Agapornis spp.</td>
<td>Droppings</td>
<td>1/13</td>
<td>Peru6&lt;sup&gt;1&lt;/sup&gt;</td>
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<td>Cockatiel/Nymphicus holandicus</td>
<td>Droppings</td>
<td>1/3</td>
<td>ND</td>
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<td></td>
<td>African grey parrot/Psittacus eritacus</td>
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<td>Peru6-var&lt;sup&gt;2&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Others</td>
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<td>Passeriformes</td>
<td>Star finch/Bathilda ruficauda</td>
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<td>Columbiformes</td>
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<td>16/44</td>
<td>Peru6</td>
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<td>1/44</td>
<td>Peru6-var</td>
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<td></td>
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<td>2/44</td>
<td>Peru6 and Peru6-var mixed infections</td>
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</table>

ND- *E. bieneusi* genotype not determined

<sup>1</sup> *E. bieneusi* genotype previously reported as Peru6 (AY371281)

<sup>2</sup> *E. bieneusi* Peru6 genotype variant with one nucleotide change (G to A) near the 3’end of the PCR fragment