Molecular Identification of *Encephalitozoon hellem* from Companion Birds Kept in Pet Shops, Japan

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ABSTRACT

To evaluate the role of companion birds as a reservoir of *Encephalitozoon hellem* infection in humans, the present study determined the prevalence and genotypes of *E. hellem* from 269 birds in 4 pet shops using polymerase chain reaction (PCR) assay. *E. hellem* was identified in 4.8% (13/269) of the birds and was detected in all pet shops. Every positive sample corresponded to zoonotic genotype 1A. Considering the low prevalence of *E. hellem* infection, it is likely that the risk of zoonotic transmission from companion birds kept in pet shops to humans is low in Japan.

**Key words**: companion bird, *Encephalitozoon hellem*, microsporidia, zoonosis

*Encephalitozoon hellem*, one of the microsporidia, is a commonly identified intracellular fungal pathogen in birds worldwide and has the potential to cause zoonotic infections. Although *E. hellem* is basically dominant in birds, this microorganism has been also commonly observed in humans. Infections with microsporidia are believed to establish via oral and/or respiratory intake of spores shed in feces and/or aerosols. In both birds and humans, *E. hellem* is an opportunistic agent that can induce a variety of clinical signs from asymptomatic to fatal. Disseminated and ocular infections with *E. hellem* have been reported in immune-deficient humans. Recently, due to the rapidly increasing elderly population suspected of having immunosuppressive status in developed countries including Japan, zoonotic transmission of *E. hellem* from birds to humans could cause significant issues.

Almost all previous studies on the prevalence of *E. hellem* infection in birds were conducted in wild birds, with only a few articles on companion birds. As companion birds are closely and frequently in contact with humans, understanding the prevalence of *E. hellem* infection is important in evaluating the risk of zoonotic transmission. Pet shops are particularly a major source of companion birds for private owners. The present study was carried out to determine the prevalence of *E. hellem* infection among companion birds kept in pet shops and the potential risk of transmission to humans in Japan.

Between March 2017 and May 2018, a total of 269 samples of freshly voided feces were randomly collected on a single occasion from individual companion birds kept in separate cages as single species in 4 pet shops (Shop 1-4) located in Kanto region, Japan. Only one fecal sample was collected when more than one bird of a single species were kept in one cage. All pet shop managers have granted permission to include their birds in the present study. The birds belonged to 44 species in 5 orders (Psittaciformes, Passeriformes, Galliformes, Columbiformes, and Anseriformes). The fecal specimens were collected immediately after natural defecation and were stored at 4°C until DNA extraction (within 3 days).

A one-step polymerase chain reaction (PCR) assay targeting the partial small subunit ribosomal RNA (SSU-rRNA) gene was employed to detect *E. hellem*. For the reaction, a forward primer (5'-TGA GAA GTA AGA TGT TTA GCA-3') and a reverse primer (5'-GTA AAA ACA CTC TCA CAC TCA -3') were used to amplify a DNA fragment of approximately 550 bp. The PCR mixture comprised of 1 × buffer containing 1.5 mM of MgCl₂, 200 µM of each dNTP, 0.
5 µM of each primer, 1.25 units of GoTaq DNA polymerase (Promega Corporation, Madison, WI, USA), and 2.0 µL of template DNA (containing approximately 10 ng of DNA) in a total reaction volume of 25 µL. The following cycling parameters were used for the reaction: after an initial denaturation for 3 min at 94°C, 35 cycles were performed; each consisting of 30 sec at 94°C for denaturation, 30 sec at 56°C for annealing, and 1 min at 72°C for extension. The samples then underwent a final extension for 5 min at 72°C. All PCR products were identified by electrophoresis on 1.5% agarose gel. After staining with AtlasSight DNA Stain (Bioatlas, Tartu, Estonia), specific DNA fragments were confirmed under UV light using a transilluminator. Incidentally, it was demonstrated that up to 10 pg DNA of E. hellem could be detected by the PCR technique used in a previous study.\(^{13}\)

PCR amplicons of the expected size were purified using a QIAquick Gel Extraction kit (QIAGEN GmbH, Hilden, Germany) and sequenced with the same primer set. Sequences were analyzed in a commercial laboratory (FASMAC Co., Ltd., Atsugi, Kanagawa, Japan). Sequence alignment and compilation were performed using the MEGA 7.026 (www.megasoftware.net) program. To determine the genotypes of E. hellem, the DNA sequences were compared with GenBank references by performing Basic Local Alignment Search Tool (BLAST) searches (http://www.ncbi.nlm.nih.gov/), and their similarity was decided based on the degree of sequence identity.

Of the 269 fecal samples from companion birds kept in pet shops, 4.8% (13 samples) were positive for E. hellem. The pathogen was detected in all 4 pet shops, at prevalence ranging from 2.5 to 8.8% (Table 1). Among the 44 examined bird species belonging to 5 orders, the companion birds carrying E. hellem belonged to 7 species from 2 orders. The prevalence of E. hellem in each of these 2 orders was 6.6% (10/152) in Psittaciformes and 3.4% (2/53) in Passeriformes, respectively. In Psittaciformes, 10 birds of 5 different species were positive for E. hellem; namely, Melopsittacus undulatus, Nymphicus hollandicus, Forpus coelestis, Agapornis roseicollis, and Agapornis fischeri (Table 2). In Passeriformes, 3 birds of 2 species were positive for E. hellem; namely, Taeniopygia guttata and Serinus canaria. Based on sequence homology, all 13 samples that were positive for E. hellem corresponded to genotype 1A with 99.62-100% similarity to accession number AF338365 (E. hellem genotype 1A small subunit ribosomal RNA gene, partial sequence) in GenBank.

The present study is the first research to determine the prevalence of E. hellem infection among companion birds kept in pet shops in Japan. Prevalence of E. hellem in companion birds based on previous epidemiological reports are as follows: 10.1% (29/287) in Czech Republic\(^9\), 15.7% (8/51) in South Korea\(^6\), 15.1% (16/106) in Brazil\(^10\), 1.1% (9/816) in

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### Table 1. Prevalence of *Enterocytozoon hellem* in companion birds kept in pet shops

<table>
<thead>
<tr>
<th>Order</th>
<th>Family</th>
<th>Shop 1</th>
<th>Shop 2</th>
<th>Shop 3</th>
<th>Shop 4</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psittaciformes</td>
<td>Psittacidae</td>
<td>7.1% (2/28)</td>
<td>5.7% (2/35)</td>
<td>3.8% (2/53)</td>
<td>6.6% (10/152)</td>
<td></td>
</tr>
<tr>
<td>Psittacidae</td>
<td>11.1% (4/36(^*))</td>
<td>8.0% (2/25)</td>
<td>3.2% (1/31)</td>
<td>3.8% (2/53)</td>
<td>5.0% (7/140)</td>
<td></td>
</tr>
<tr>
<td>Psittacinae</td>
<td>40.0% (2/5)</td>
<td>0% (0/3)</td>
<td>25.0% (1/4)</td>
<td>-</td>
<td>25.0% (3/12)</td>
<td></td>
</tr>
<tr>
<td>Psittacinae</td>
<td>7.7% (1/13)</td>
<td>8.0% (2/25)</td>
<td>0% (0/26)</td>
<td>0% (0/25)</td>
<td>3.4% (3/89)</td>
<td></td>
</tr>
<tr>
<td>Psittacinae</td>
<td>0% (0/7)</td>
<td>11.1% (2/18)</td>
<td>0% (0/16)</td>
<td>0% (0/25)</td>
<td>3.0% (2/66)</td>
<td></td>
</tr>
<tr>
<td>Psittacinae</td>
<td>20.0% (1/5)</td>
<td>0% (0/6)</td>
<td>0% (0/10)</td>
<td>-</td>
<td>4.8% (1/21)</td>
<td></td>
</tr>
<tr>
<td>Sturnidae</td>
<td>0% (0/1)</td>
<td>0% (0/1)</td>
<td>-</td>
<td>0% (0/2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galliformes</td>
<td>0% (0/8)</td>
<td>-</td>
<td>0% (0/14)</td>
<td>-</td>
<td>0% (0/22)</td>
<td></td>
</tr>
<tr>
<td>Phasianidae</td>
<td>0% (0/8)</td>
<td>-</td>
<td>0% (0/14)</td>
<td>-</td>
<td>0% (0/22)</td>
<td></td>
</tr>
<tr>
<td>Colubiformes</td>
<td>-</td>
<td>0% (0/2)</td>
<td>0% (0/3)</td>
<td>-</td>
<td>0% (0/5)</td>
<td></td>
</tr>
<tr>
<td>Columbidae</td>
<td>-</td>
<td>0% (0/2)</td>
<td>0% (0/3)</td>
<td>-</td>
<td>0% (0/5)</td>
<td></td>
</tr>
<tr>
<td>Anseriformes</td>
<td>-</td>
<td>-</td>
<td>0% (0/1)</td>
<td>-</td>
<td>0% (0/1)</td>
<td></td>
</tr>
<tr>
<td>Anatidae</td>
<td>-</td>
<td>-</td>
<td>0% (0/1)</td>
<td>-</td>
<td>0% (0/1)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8.8% (5/57)</td>
<td>7.3% (4/55)</td>
<td>2.5% (2/79)</td>
<td>2.6% (2/78)</td>
<td>4.8% (13/269)</td>
<td></td>
</tr>
</tbody>
</table>

\(^{-}\): Not included \(^{*}\): Positive number/Examined number
suspected to be the main factor influencing the difference in level of environmental contamination with sanitary control in each facility was not evaluated here, the prevalence, namely, 2.5 to 8.8%, among pet shops. Although shops examined. The present study revealed a wide range of prevalence between reports requires careful considerations due to the difference in research populations, including scale, bird species, and regions, the present study suggests that there is no predisposition for E. hellem infection based on bird order, and further investigations are needed to clarify this hypothesis.

In the present study, all 13 isolates determined positive for E. hellem were identified as genotype 1A based on the sequence homology. Due to having many cases of genotype 1A isolates, only a few cases with 2C, and rare cases of 2B in birds, E. hellem genotype 1A is considered as the predominant genotype in birds. However, genotype 1A was often isolated from immune-deficient human patients. Hence, this suggests that birds are an important reservoir for zoonotic transmission of E. hellem. In addition, since E. hellem infection in birds is not always associated with clinical disorders, pet shop staff and bird owners may be unaware that their environment is contaminated with feces and aerosols from infected companion birds. Preventing E. hellem infection from companion birds requires adequate sanitary management, such as rapid elimination of voided feces and regular ventilation of room air. Nevertheless, considering the low-level prevalence of E. hellem infection, it is likely that the risk of zoonotic transmission from companion birds kept in pet shops to humans is low in Japan.

**Conflicts of interest**

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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5) Black SS, Steinohrt LA, Bertucci DC, Rogers LB, Didier ES: Encephalitozoon hellem in budgerigars (Melopsittacus undula-

<table>
<thead>
<tr>
<th>Order</th>
<th>Family</th>
<th>Species</th>
<th>Percentage of Isolated Samples</th>
<th>Examined Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psittaciformes</td>
<td>Psittacidae</td>
<td>Melopsittacus undulatus</td>
<td>6.8% (4/59*)</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Forpus coelestis</td>
<td>33.3% (1/3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Agapornis roseicollis</td>
<td>3.4% (1/29)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Agapornis fischeri</td>
<td>100% (1/1)</td>
<td></td>
</tr>
<tr>
<td>Cacatudae</td>
<td></td>
<td>Nymphicus hollandicus</td>
<td>33.3% (3/9)</td>
<td></td>
</tr>
<tr>
<td>Passeriformes</td>
<td>Estrildidae</td>
<td>Taeniopygia guttata</td>
<td>10.0% (2/20)</td>
<td></td>
</tr>
<tr>
<td>Fringilidae</td>
<td></td>
<td>Serinus canaria</td>
<td>6.3% (1/16)</td>
<td></td>
</tr>
</tbody>
</table>

*: Positive number/Examined number

Iran, and 14.7% (21/143) in Turkey. The results of these studies show a higher prevalence compared with the present results, except for the report from Iran. Although comparing prevalence between reports requires careful considerations due to the difference in research populations, including scale, bird species, and regions, the present study suggests that E. hellem infection has low prevalence among companion birds kept in pet shops in Japan. Widespread infection, however, is suspected because the pathogen was confirmed in all the 4 pet shops examined. The present study revealed a wide range of prevalence, namely, 2.5 to 8.8%, among pet shops. Although sanitary control in each facility was not evaluated here, the level of environmental contamination with E. hellem is suspected to be the main factor influencing the difference in prevalence among pet shops. Companion birds in pet shops are usually kept in limited spaces in shop facilities. Additionally, multiple individuals of the same species are often housed in a single cage. Consequently, the birds live in significantly close distance from each other. It is likely that such environments facilitate the transmission of E. hellem through contaminated food and water. The inhalation of aerosol containing microsporidian spores, which are dispersed aerially, is also suggested as a significant factor for transmission in contaminated environments. Therefore, it is difficult to eliminate transmission among companion birds in facilities such as pet shops.

E. hellem infection was determined in 7 bird species belonging to 2 orders of Psittaciformes and Passeriformes. As Psittaciformes and Passeriformes are major companion birds, previous studies have also found E. hellem frequently from these 2 orders. Infection with E. hellem could not be confirmed in the other 3 orders, namely, Columbiformes, Galliformes, and Anseriformes; although the number of examined birds is insufficient. To our knowledge, no reports have yet been published regarding the determination of E. hellem infection from companion birds in these 3 orders. According to the previous studies in wild birds, however, E. hellem infections have also been demonstrated in birds belonging to orders Columbiformes, Galliformes, and Anseriformes, among others. Therefore, it is likely that there is no predisposition for E. hellem infection based on bird order, and further investigations are needed to clarify this hypothesis.

The present study revealed a wide range of prevalence, namely, 2.5 to 8.8%, among pet shops. Although comparing prevalence between reports requires careful considerations due to the difference in research populations, including scale, bird species, and regions, the present study suggests that there is no predisposition for E. hellem infection based on bird order, and further investigations are needed to clarify this hypothesis.

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