

DNA Detection and Genotypic Identification of Potentially Human-Pathogenic Microsporidia from Asymptomatic Pet Parrots in South Korea as a Risk Factor for Zoonotic Emergence[∇]

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We detected and identified genotypes of human-pathogenic microsporidia in fecal samples from 51 asymptomatic captive-bred pet parrots in South Korea. Microsporidia were identified in 8 samples (15.7%); 7 parrots tested positive for *Encephalitozoon hellem*, and 1 parrot tested positive for both *E. hellem* and *Encephalitozoon cuniculi*. In genotypic identifications, *E. hellem* was present in genotypes 1A and 2B and *E. cuniculi* was present in genotype II. Pet parrots might be a source of human microsporidian infection.

Microsporidia are obligate, intracellular, eukaryotic, single-celled fungi that infect a wide variety of vertebrate and invertebrate hosts (5). Microsporidiosis is increasingly recognized as an opportunistic disease in immunocompromised and immunocompetent individuals (5). To date, among the eight microsporidian species identified in humans, *Enterocytozoon bineusi*, *Encephalitozoon intestinalis*, *Encephalitozoon hellem*, and *Encephalitozoon cuniculi* are newly emerging zoonotic pathogens. *E. bineusi* and *E. intestinalis* induce chronic diarrhea in immunocompromised patients and induce acute self-limiting diarrhea in immunocompetent persons. *E. hellem* and *E. cuniculi* have been less commonly diagnosed and cause ocular or disseminated infections in immunocompromised patients (15).

All microsporidian species that infect humans have also been detected in various bird species. Microsporidiosis in birds was first described in masked lovebirds (*Agapornis personta*) in 1975 (13). After identification of microsporidia in birds, birds were considered the primary hosts of *E. hellem*, and droppings of asymptomatic pet birds were considered a potential environmental source of *E. hellem* infection in humans (2). In captive birds, microsporidiosis caused by *E. hellem* was first reported in budgerigars (*Melopsittacus undulatus*) in 1997 (3). Since then, numerous cases have been reported in a variety of pet psittacine birds (4, 16, 17). The less common microsporidian species, *E. bineusi* and *E. cuniculi*, have also been detected in psittacine birds, but *E. intestinalis* has been detected only in a few birds (11, 14). *Encephalitozoon* infections in birds typically present nonspecific signs, such as depression, decreased appetite, weight loss, and diarrhea, and unusually show ocular signs, such as conjunctivitis and keratitis. However, many in-

fecting birds present as an unexpected death without clinical signs (20). The purpose of this study was to evaluate the prevalence of microsporidia in fecal samples of asymptomatic parrots kept in private households in South Korea.

Sample collection. A total of 51 fresh fecal samples were collected from parrots which were bred in captivity and kept in private households. All birds surveyed appeared healthy at the time of sampling. Samples were collected by placing paper sheets under the cages of birds. Fresh droppings of birds were placed in sterile tubes and frozen immediately at -70°C until analysis.

PCR and genotyping. To extract DNA from fecal samples, the samples were resuspended in phosphate-buffered saline (PBS) (pH 7.0) and then mixed thoroughly by vortexing. The supernatants were used to extract nucleic acid. DNAzol (Invitrogen, Karlsruhe, Germany) was used according to the manufacturer's instructions, and the nucleic acid was eluted with 50 μl of Tris-EDTA (TE) buffer. For identification of microsporidian species in fecal samples, primer sets were used as previously published (12). Nested PCR was carried out using the Maxime PCR premix kit (Intron Biotechnology Inc., Seongnam, South Korea). Each product was examined via electrophoresis in 2% agarose gels stained with ethidium bromide. The PCR products were sequenced with ABI PRISM BigDye terminator v3.0 cycle sequencing kit (Applied Biosystems, Foster City, CA). Each PCR product was genetically identified through DNA homology analyses of a nucleotide sequence database using the BLAST algorithms, and subspecies and genotypes were identified.

Detection and genotype identification of *Encephalitozoon* spp. No clinical signs of microsporidiosis were observed in any bird examined in this study. Eight samples of a total of 51 (15.7%) tested positive. *E. hellem* was detected in 7 birds (13.7%). Coinfection with *E. cuniculi* and *E. hellem* was detected in 1 bird (2.0%). No *E. intestinalis* and *E. bineusi* were detected. To identify genotypes of microsporidian species, sequences of the positive samples were compared with the ref-

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TABLE 1. Differentiation and genotype identification of isolated microsporidian species in fecal samples from pet parrots

Species and genotype	No. of positive samples (n = 51)	Prevalence (%)
<i>E. cuciculi</i> genotype II	1 ^a	2.0 ^a
<i>E. hellem</i> genotype 1A	7	13.7
<i>E. hellem</i> genotype 2B	1 ^a	2.0 ^a
Total	8	15.7

^a One bird was found to be coinfecting with *E. cuciculi* and *E. hellem*.

reference genotype from GenBank. *E. hellem* was present in two different genotypes, genotypes 1A and 2B, and *E. cuciculi* was present in one genotype, genotype II (Table 1). The close relatedness between the isolates was also reflected in the phylogram (Fig. 1). The sequence homology was about 97 to 99% compared with reference strain of *E. hellem* genotype 1A (GenBank accession number AF338367.1). However, the nucleotide sequence homology of genotype 2B was comparatively low to the reference genotypes from GenBank, 90% with reference strain of *E. hellem* genotype 2B (GenBank accession number AF338368.1). This was suspected to be due to the low quality of the template DNA. In the case of *E. cuciculi* genotype II, the sequence homology was about 95% (GenBank accession number EU001241.1). In the study population, 6 new psittacine bird hosts were found: African gray parrot (*Psittacus erithacus*), blue-streaked lory (*Eos reticulata*), chestnut-fronted macaw (*Ara severus*), eclectus (*Eclectus roratus*), green-cheeked parakeet (*Pyrrhura molinae*), red-shouldered macaw (*Diopsittacus nobilis*), and rose-ringed parakeet (*Psittacula krameri*).

Discussion. Because microsporidia are important opportunistic pathogens in humans, more research must be done to diagnose microsporidiosis and to determine the potential host range. Diagnostic procedures such as specific stains are not readily available in diagnostic facilities due to their low detection limit (7, 11). Molecular methods have been used to diagnose microsporidiosis, and these methods are more sensitive in the diagnosis of spore shedding in naturally infected asymptomatic birds (6, 18). Currently, molecular methods are used to identify potentially human-pathogenic microsporidian genotypes in birds, since they are a potential environmental source of microsporidian infection (2, 8, 10, 11, 14).

Using a molecular method, we detected microsporidia in 15.7% of fecal samples from pet parrots in South Korea, which is a relatively low prevalence. Recently reported prevalences of microsporidia were 11%, 28.9%, and 40.1% in various birds in The Netherlands, Portugal, and the Czech Republic, respectively (1, 11, 14). The most prevalent microsporidian species in this study was *E. hellem* (13.7%). *E. bieneusi* and *E. intestinalis* were not detected. This was an expected result, because birds were considered to be a primary host of *E. hellem* (15). Barton et al. also suggested that the most frequent microsporidia in birds is *E. hellem* (2). However, recent studies reported that *E. hellem* was the least frequently identified microsporidia and that *E. bieneusi* was assumed to be the most frequent microsporidia in birds (1, 11, 14).

By sequencing the positive products using the internal transcribed spacer (ITS) gene, two genotypes from *E. hellem*, genotype 1A and genotype 2B, were identified. Genotype 1A

(9.8%) was more common than genotype 2B (2.0%). Genotype 1 is the most commonly identified subtype in humans and birds (19). Although genotype 1A was previously reported in birds (11), genotype 2B has so far not been identified in birds. Genotype 2B has been reported from a patient in Switzerland and is thought to be a genotype derived from humans (21). According to previous studies analyzing *E. hellem* genotypes, isolates from humans show regional differences, and isolates from birds have not been subjected to these types of genetic analysis. These results suggest the existence of extensive genetic diversity in *E. hellem* isolates between humans and birds worldwide.

Rabbits and rodents are the main hosts of *E. cuciculi*, but two recent reports suggest that birds may be a new host of *E. cuciculi* (1, 11). In this study, one *E. cuciculi* genotype, genotype II (2.0%), was identified from one bird. Each *E. cuciculi* genotype has a specific host preference and geographical distribution. Genotype II is classified as a mouse strain and has been identified only in Europe. Genotype I was identified in rabbits in America, Australia, and Europe, and genotype III infects dogs from America, South Africa, and Europe (15). *E. cuciculi* genotype II isolation from birds in Korea supports the expansion of its host diversity and geographical distribution. Genotypes I and III have been reported in humans, but genotype II has not been reported. However, *E. cuciculi* infection may occur in imported animals from other countries and may subclinically infect animals (9). Potential spreading of genotypes not previously identified in humans should be considered.

The results of the present study demonstrated that microsporidia are widespread in asymptomatic psittacine birds kept in private households. The relatively high prevalence in pet parrots suggests that pet parrots might be a potential source of human infection and environmental contamination in South Korea. Moreover, genotypic analysis of microsporidia implies the existence of extensive genetic diversity between

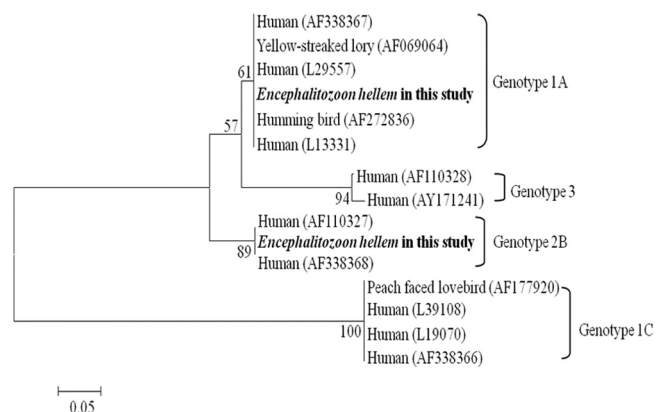


FIG. 1. Phylogenetic tree for nucleotide sequences of *Microsporidium hellem* from pet parrots examined in the present study compared to other genotypes based on internal transcribed spacer (ITS) ribosomal DNA (rDNA) sequence. GenBank accession numbers are shown in parentheses. The evolutionary relationships of these genes were generated by the neighbor-joining (NJ) method with a bootstrap value of 1,000 using MEGA4 software. The numbers at the nodes represent the percentage with a bootstrap value of 1,000. Bar, 0.05 substitution per site.

humans and birds and the expansion of host diversity and geographical distribution.

Nucleotide sequence accession number. The unique *E. hellem* ITS sequence obtained in this study has been deposited in GenBank under accession number JF836368.

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