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Frequent Occurrence of Human-Associated Microsporidia in Fecal Droppings of Urban Pigeons in Amsterdam, The Netherlands[▽]

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Human-associated microsporidia were frequently observed in fecal samples of 331 feral pigeons in Amsterdam, The Netherlands, obtained during high- and low-breeding periods. Thirty-six of 331 samples (11%) contained the human pathogens *Enterocytozoon bieneusi* ($n = 18$), *Encephalitozoon hellem* ($n = 11$), *Encephalitozoon cuniculi* ($n = 6$), and *Encephalitozoon intestinalis* ($n = 1$); 5 samples contained other microsporidia. Pigeon feces can be an important source of human microsporidian infection.

The microsporidia form a diverse group of organisms consisting of more than 1,200 species in various vertebrate and invertebrate hosts. Four species emerged as frequent and important opportunistic pathogens when AIDS became pandemic: *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *Encephalitozoon hellem*, and *Encephalitozoon cuniculi* (2). The high seroprevalence against *Encephalitozoon* species in Dutch blood donors and French women suggested that contact with *Encephalitozoon* spp. is also common in immunocompetent subjects (14). Animal reservoirs (10), surface water (12), and humans (1) are thought to be sources of human infection. However, which animal species are relevant reservoirs or sources is still unclear.

In many European cities, the feral pigeon (*Columba livia* [forma *domestica*]) is an abundant bird species that often lives in close contact with humans. Recently, *E. bieneusi* and other microsporidia were detected in fecal droppings of these pigeons (5, 9). The objective of the present study was to investigate the presence of microsporidia in urban feral pigeons in Amsterdam.

DNA was isolated from 331 pigeon fecal samples from 331 pigeons obtained during a study of *Chlamydophila psittaci*, as described by Heddema et al. (7, 12). Briefly, at nine locations, pigeons were attracted with food, and their fresh fecal droppings were collected from pavements by using sterile cotton swabs (MW&E, United Kingdom) during a low-breeding period (between 3 February and 8 March 2005) and when breeding was frequent (2 May 2005). The cotton swabs were placed in 1.5-ml tubes in 300 μl of Baker water (Boom B.V., Meppel, The Netherlands) and vortexed thoroughly. Fifty microliters of each fecal suspension was used as input for the DNA extraction procedure using a modified Boom extraction (6) or a High Pure DNA purification kit (Roche). PCR inhibition was excluded by amplification of a control target, as previously described (7). PCR was performed using the primers and conditions described by Notermans et al. (11), directed to a conserved region of the small-subunit rRNA gene of microsporidia. PCR products were size separated by agarose gel electrophoresis and visualized by UV illumination after ethidium bromide staining. A unidirectional work-flow was maintained with separation of the PCR mixture preparation and the DNA extraction from all (post) amplification activities, and negative controls were included in all steps of the detection process. Amplicons were sequenced using BigDyeTerminator chemistry (Applied Biosystems) and analyzed on an ABI 3900 sequencer. Resulting sequences were analyzed using CodonCode Aligner (CodonCode Corp.) and MEGA (13) software.

Sequence-confirmed microsporidium-positive PCR products were obtained at all sampling locations for 41/331 samples (12%) (Table 1). In the low-breeding period, 6% (10/160; 95% confidence interval [CI], 2 to 10) of the samples were positive, whereas during the breeding period, this number increased significantly to 18% (31/171; 95% CI, 12 to 24; Fisher's exact test, $P = 0.001$). This indicates that either infection with mi-

TABLE 1. Number of microsporidium-positive feral pigeon fecal samples by location in Amsterdam, The Netherlands

Town council	No. of positive samples/total no. of samples	
	Low-breeding period	Breeding period
Oost Watergraafsmeer	2/15	2/15
Oud Zuid	0/15	6/20
Binnenstad (Dam)	1/20	4/27
Binnenstad (Leidseplein)	4/25	2/27
Zeeburg	0/15	4/15
Zuider Amstel	1/15	5/15
Geuzenveld	0/15	4/15
Bos en Lommer	0/20	1/15
Oud West	2/20	3/22
Total	10/160	31/171

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TABLE 2. Sequence diversity in PCR positive samples

No. of 18S sequences	GenBank accession no.	No. of identical nucleotides/ total no. of nucleotides (%)	Most similar GenBank entry	Species	Human isolate
18	EU828594	239/240	L16868	*Enterocytozoon bieneusi*	+
6	EU828595	243/243	X98470	*Encephalitozoon cuniculi*	+
11[a]	EU828596	249/249	AF338364	*Encephalitozoon hellem*	+
1	EU828597	240/240	SIU09929	*Encephalitozoon intestinalis*	+
1	EU828591	200/211 (94)	U11046	*Vittaforma corneae*	+
2	EU828592	199/212 (93)	AY502945	*Endoreticulatus* sp.	−
1	EU828590	199/212 (93)	AY233131	*Cystosporogenes legeri*	−
1	EU828593	(<90)			−
1[a]	EU828598	(<90)			−

[a] One isolate had a mixed infection.

crosporidia is transient and fluctuates over time or that shedding occurs intermittently. The latter case might be activated by the physiological changes related to breeding that impact behavior and immune status, as has been observed for other pathogens (e.g., *C. psittaci*) (7). In the present population, microsporidian infection was more frequent than *C. psittaci* infection. No significant relationship between *Chlamydophila* positivity and microsporidian positivity of samples was observed.

The 41 positive samples contained eight different sequences. Sequence accession numbers and similarities to GenBank entries are given in Table 2. Thirty samples showed sequences identical or highly similar to those of the human pathogens *Enterocytozoon bieneusi*, *Encephalitozoon hellem*, and *E. intestinalis*. Surprisingly, six samples contained sequences identical to that of *E. cuniculi*. This species has been detected in various mammalian species (10) but only once in a captive bird (8). One sample had a sequence that was 94% similar to that of *Vittaforma corneae*. Since the similarity between the 12 different *V. corneae* entries in GenBank is higher, it is unclear whether this sequence represents DNA from *V. corneae* or from a closely related but different species. The four remaining sample sequences were similar to those of microsporidia that are not pathogens of humans; one sample contained both an *E. hellem* sequence and a sequence of another microsporidium. The relative low similarity scores obtained for these sequences probably reflect the underrepresentation of nonpathogenic, environmental microsporidia in GenBank.

Our data show the presence of *Enterocytozoon bieneusi*, *Encephalitozoon hellem*, *E. intestinalis*, and *E. cuniculi* in feces of urban feral pigeons from Amsterdam. It confirms and extends the data from Lobo et al. (9), who found *E. bieneusi* in urban pigeons. To our knowledge, the observation of *Encephalitozoon* species in pigeons has appeared in only one report (5), i.e., *E. hellem* and *E. intestinalis* species in fecal samples of pigeons in Murcia (Spain). No *E. cuniculi* was detected in that study, whereas in our samples, this was the third most abundant microsporidian species. Whether the differences in relative abundance of the different species are due to differences in pigeon population, time of isolation, geographical location, or targets used for detection and identification (small-subunit RNA versus internal transcribed spacer) remains to be investigated.

Since the variation of microsporidian species in pigeons is unknown, we employed a PCR-and-sequencing combination that also allowed for amplification of DNA from nonpathogenic microsporidia. Whether the microsporidia that were encountered represent a "pass through" situation, where pigeons ingested small numbers of spores while eating, or a true "infection" remains to be assessed. However, the detection of *Enterocytozoon bieneusi*, *Encephalitozoon cuniculi*, and *E. hellem* DNA in multiple samples of different pigeons suggests true infection.

The minimal infectious dose for microsporidia is unknown but is thought to be comparable to those of other intestinal parasites, i.e., 10 to 100 spores (3). In a quantitative study of pigeon guano, Graczyk et al. found 3,500 *E. bieneusi* spores/g (4). It was calculated that a person with a 30-min exposure to pigeon guano through nearby sweeping with a broom could inhale >1,000 viable *E. bieneusi* spores. Close interaction of feral pigeons with humans (especially children and elderly persons and tourists) is common in Amsterdam and is higher during the breeding period than the low-breeding period. Also, pigeon nests that are built near ventilation inlets could result in the inhalation of spores.

Since microsporidia are important opportunistic pathogens, the risk of such exposure can be of significance for the immunocompromised human host. Frequent exposure to bird droppings might partly explain the observed high seroprevalence against *Encephalitozoon* species in Dutch blood donors (14).

In conclusion, this study supports the finding that microsporidiosis is a zoonotic disease (10) and suggests that excreta of urban feral pigeons can be an important source of human infection with *Enterocytozoon bieneusi*, *Encephalitozoon hellem*, *E. intestinalis*, and also *E. cuniculi*.

Nucleotide sequence accession numbers. The sequences reported in the manuscript are available through GenBank accession numbers EU828590 to EU828598.

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