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Microsporidian Species Known To Infect Humans Are Present in Aquatic Birds: Implications for Transmission via Water?

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Human microsporidiosis, a serious disease of immunocompetent and immunosuppressed people, can be due to zoonotic and environmental transmission of microsporidian spores. A survey utilizing conventional and molecular techniques for examining feces from 570 free-ranging, captive, and livestock birds demonstrated that 21 animals shed microsporidian spores of species known to infect humans, including *Encephalitozoon hellem* (20 birds; 3.5%) and *Encephalitozoon intestinalis* (1 bird; 0.2%). Of 11 avian species that shed *E. hellem* and *E. intestinalis*, 8 were aquatic birds (i.e., common waterfowl). The prevalence of microsporidian infections in waterfowl (8.6%) was significantly higher than the prevalence of microsporidian infections in other birds (1.1%) ($P < 0.03$); waterfowl fecal droppings contained significantly more spores (mean, 3.6×10^5 spores/g) than nonaquatic bird droppings contained (mean, 4.4×10^4 spores/g) ($P < 0.003$); and the presence of microsporidian spores of species known to infect humans in fecal samples was statistically associated with the aquatic status of the avian host ($P < 0.001$). We demonstrated that a single visit of a waterfowl flock can introduce into the surface water approximately 9.1×10^8 microsporidian spores of species known to infect humans. Our findings demonstrate that waterborne microsporidian spores of species that infect people can originate from common waterfowl, which usually occur in large numbers and have unlimited access to surface waters, including waters used for production of drinking water.

Microsporidians are obligate intracellular parasites that are emerging opportunistic pathogens that infect both immunocompromised and immunocompetent people (41, 42, 43). Microsporidians are on the Contaminant Candidate List of the U.S. Environmental Protection Agency because their transmission routes are unknown, spore identification, removal, and inactivation in drinking water are technologically challenging (27), and human infections are difficult to treat (11). Considerable evidence gathered to date indicates that water is involved in the epidemiology of human microsporidiosis (10, 12, 14, 15, 25, 35, 38); however, this epidemiological link has not been proved conclusively (16).

Identification of microsporidian spores of species known to infect humans (*Encephalitozoon intestinalis*, *Encephalitozoon hellem*, *Encephalitozoon cuniculi*, and *Enterocytozoon bieneusi*) is a challenge because microsporidians can infect a variety of nonhuman hosts and spore morphology does not provide enough information for species identification (39). This challenge is met by the multiplex fluorescence in situ hybridization (FISH) assay, which employs fluorescently labeled oligonucleotide probes targeted to species-specific sequences of 18S rRNA (19, 20, 22). By using various fluorochromes to label different oligonucleotide probes, spores of *E. intestinalis*, *E. hellem*, *E. cuniculi*, and *E. bieneusi* are stained red, green, orange, and yellow, respectively (19, 20, 22).

E. hellem has been found most frequently in avian hosts (5, 7, 8, 26, 28, 32, 33, 34, 37), and based on these reports it has commonly been assumed that there is a zoonotic threat (8, 21), although there is no direct evidence of this. Most of the reports of *E. hellem* are anecdotal, related to infection of a single bird or a few companion or domestic birds (5, 7, 26, 32, 34, 37), and the epidemiological implications are limited. Although identification of *E. hellem* in these reports left no doubt about species identity, it is difficult to explain the increased number of human microsporidiosis cases (43) by contact with companion birds. Additionally, people with immunological impairments are usually aware of potential infection hazards from pet animals (40, 41, 42). As demonstrated recently, exposure to urban park pigeons might be an important link in the epidemiology of zoonotic microsporidiosis, particularly in children and elderly people (21). However, the possibility of waterborne transmission of microsporidian spores, although seriously considered by agencies concerned with drinking water quality (27), has not been appropriately addressed yet through epidemiological studies. Microsporidian spores have been detected in a variety of surface waters (3, 12, 15, 20, 35, 38), and water has been implicated as a source of human infection based on epidemiological data (10, 16). Although the actual route of transmission of *E. hellem* spores is not known, it is quite plausible that

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TABLE 1. Results of testing 570 bird fecal dropping samples for microsporidian spores of species that are known to infect humans using conventional stains (Chromotrope-2R and calcofluor white M2R) and multiplex FISH

Host species[a]	Samples			Microsporidian spores	
	Total no.	No. positive	% Positive	Species	Mean concn (spores/g of stool)
Mallard duck (*Anas platyrhynchos*)[b]	28	5	18	*E. hellem*	3.8×10^5
Greyleg goose (*Anser anser*)[b]	34	3	9	*E. hellem*	2.6×10^5
Mute swan (*Cygnus olor*)[b]	30	4	13	*E. hellem*	5.1×10^5
Carrion crow (*Corvus corone*)	51	1	2	*E. hellem*	3.1×10^4
Budgerigar (*Melopsittacus undulatus*)	1	1	100	*E. hellem*	2.0×10^3
Nicobar pigeon (*Caloenas nicobarica*)	2	1	50	*E. hellem*	1.0×10^5
Black swan (*Cygnus atratus*)[b]	4	1	25	*E. hellem*	4.0×10^5
Black-necked swan (*Cygnus melanocoryphus*)[b]	4	2	50	*E. hellem*	3.1×10^5
Coscoroba swan (*Coscoroba coscoroba*)[b]	1	1	100	*E. hellem*	2.0×10^5
Black-crowned crane (*Balearica pavonina*)[b]	4	1	25	*E. hellem*	2.0×10^4
Domestic goose (*Anser anser domestica*)[b]	11	1	9	*E. intestinalis*	4.0×10^5

[a] *Anas platyrhynchos*, *Anser anser*, *Cygnus olor*, and *Corvus corone* were free-ranging birds; *Melopsittacus undulates*, *Caloenas nicobarica*, *Cygnus atratus*, and *Cygnus melanocoryphus* were captive birds; and *Anser anser domestica* was a livestock bird. The results for only positive fecal samples and host species are shown.
[b] Waterfowl species.

waterborne microsporidian spores can originate from aquatic birds; however, this possibility has not been investigated previously. Therefore, we initiated an intensive, long-term monitoring study of a variety of ecologically diverse groups of free-ranging, captive, and domestic livestock birds in order to characterize the potential input of human-infectious microsporidian spores from avian hosts.

MATERIALS AND METHODS

A total of 570 fecal samples were collected from randomly selected birds, including 224 free-ranging birds, 115 captive birds (99 birds from a zoological garden and 16 birds from pet stores), and 231 livestock birds. The samples were obtained in northwest Poland. The birds represented 57 species, including 13 free-ranging species, 45 captive species, and three domestic species. The families of the free-ranging birds included the Anatidae, Ardeidae, Ciconiidae, Laridae, Motacillidae, Corvidae, Accipitridae, and Falconidae. The captive birds were members of the Psittacidae, Columbidae, Corvidae, Fringillidae, Sturnidae, Phasianidae, Cracidae, Rhamphastidae, Bucerotidae, Ciconiidae, Threskiornithidae, Phoenicopteridae, Anatidae, Anhimidae, and Gruidae. The domestic livestock birds included members of the Phasianidae and Anatidae. Free-ranging bird samples were obtained from 10 sites, samples from captive birds were obtained from one zoo and four pet stores, and livestock bird samples were obtained from three commercial, government-run farms. Fecal samples were collected just after defecation, placed in sterile plastic tubes, transported to the laboratory in a cooler, and stored at 4°C until they were analyzed. Efforts were made to collect a single fecal specimen from a single bird, and the entire dropping material was used for processing.

In the laboratory, direct wet smears were prepared from all fecal samples in duplicate and then stained with Chromotrope-2R and calcofluor white M2R (2, 11). The smears were examined by light microscopy using a ×100 oil immersion objective, and a sample was considered positive if a single microsporidian spore was detected. Approximately 1.5 g of a fecal specimen was emulsified with 3 ml of sterile phosphate-buffered saline (PBS) (pH 7.4) and processed by sugar-phenol flotation (2). One milliliter of the top layer was collected and centrifuged (8,000 × *g*, 5 min), and the supernatant was discarded. The pellet was resuspended in 100 μl of sterile PBS.

The samples were coded, and multiplex FISH assays for *E. intestinalis*, *E. hellem*, *E. cuniculi*, and *E. bieneusi* were carried out in Eppendorf tubes by using 100 μl (total volume) of hybridization buffer at 57°C for 3 h (19, 20, 22). After hybridization, the tubes were centrifuged twice at 4°C (2,000 × *g*, 5 min), and the pellets were resuspended in 100 μl of sterile PBS. Five 20-μl samples were transferred into five lysine-coated wells (diameter, 5 mm) on a Teflon-coated glass slide (Carlson Scientific, Inc., Protone, Ill.) and air dried. The entire area of a well was examined with an Olympus BH2-RFL epifluorescence microscope by using a dry ×60 objective and a BP450-490 exciter filter, and the spores were enumerated. A sample was considered positive if a single microsporidian spore was detected. The samples were uncoded, and the FISH results were compared to the results obtained by conventional stain testing.

A statistical analysis was carried out with Statistix 7.0 (Analytical Software, St. Paul, MN). Variables were tested with Wilk-Shapiro ranking plots to determine whether the distribution conformed to a normal distribution; if it did not, a nonparametric test was used. The results were expressed as the mean ± standard deviation for continuous variables and as the number or percentage for categorical data. The statistical significance of the association between the type of bird (i.e., aquatic or nonaquatic) and the presence of microsporidian spores in the feces was assessed by Fisher's exact test. Statistical significance between fractions was assessed by a chi-square test. Statistical significance was defined as a P value of <0.05, and all P values were two tailed.

RESULTS

The spores stained pinkish when Chromotrope-2R was used and had the characteristic morphology of microsporidian spores with a clear vacuole-like polar end; they were ovoid and ranged from 0.9 to 1.6 μm long. Using FISH, spores of *E. hellem* were identified in 20 of 570 fecal samples (3.5%), and *E. intestinalis* was identified in a single fecal sample (0.2%) (Table 1). All samples identified as positive by FISH were also positive as determined with Chromotrope-2R and calcofluor white M2R. However, two samples identified as positive by calcofluor white M2R staining were negative as determined by Chromotrope-2R staining and FISH. FISH-processed *E. hellem* spores stained bright green, and *E. intestinalis* spores stained bright red; both species exhibited typical microsporidian morphology, with more intense fluorescent staining in the polar half of the spore.

Of 570 fecal samples, 21 (3.7%) contained variable numbers of microsporidian spores as determined with conventional stains (Chromotrope-2R and calcofluor white M2R) and by FISH (Table 1). A total of 13 of 224 (5.8%) free-ranging birds, 7 of 115 (6.1%) captive birds, and 1 of 231 (0.4%) livestock birds had microsporidian spores of species that are known to infect humans in their feces. The prevalence of spore-positive samples was significantly lower for livestock birds than for free-ranging or captive birds ($P < 0.01$; $F = 6.6$; chi-square test).

The prevalence of spore-positive samples for the avian host species varied from 2 to 100%, and the mean prevalence was 36.6% ± 34.9% (Table 1). A total of 11 bird species had

microsporidian spores of species that are known to infect humans in their fecal samples, and 8 of these species were aquatic birds (common waterfowl species) (Table 1). The prevalence of microsporidian infection in waterfowl (8.6%) was significantly higher than the prevalence of microsporidian infection in other birds (1.1%) ($P < 0.03$; $F = 4.9$; chi-square test). As demonstrated by the Fisher exact test, the presence of microsporidian spores in the fecal samples was statistically strongly associated with the aquatic status of the avian host species ($P < 0.001$; $F = 20.09$).

The overall concentration of human-infectious microsporidian spores in bird fecal droppings was $3.1 \times 10^5 \pm 1.9 \times 10^5$ spores per g (Table 1). Feces originating from waterfowl contained significantly more spores (mean, 3.6×10^5 spores/g) than feces from nonaquatic birds contained (mean, 4.4×10^4 spores/g) ($P < 0.003$; $t = 2.47$; Wilcoxon rank sum test) (Table 1).

DISCUSSION

The present study demonstrated that (i) *E. hellem* was the predominant microsporidian species in birds; (ii) the species that cause the vast majority of avian microsporidiosis cases and are known to infect people were found in common waterfowl that are usually abundant in the wild; and (iii) the number of microsporidian spores excreted by birds was generally high and was significantly higher in birds having frequent contact with surface water. As shown here, waterborne transmission of microsporidian spores should be seriously considered in the epidemiology of human microsporidiosis and should be appropriately addressed by agencies and institutions concerned with drinking water quality. Microsporidian spores have been found in surface waters; however, unfortunately, in some of the studies the species were not identified (3, 15). Also, risk factors for intestinal microsporidiosis suggest that water is a source of infection (15, 16). In addition, the present study demonstrated that both free-ranging birds and captive birds can be a source of microsporidian spores of species known to infect humans. Thus, the concept postulated previously that companion and pet birds can represent a zoonotic microsporidiosis threat is valid (40, 41, 42, 43).

It is difficult to compare the overall prevalence of avian microsporidiosis demonstrated in the present study (3.7%) with other data, because most of the studies of *E. hellem* in birds have been based on single or selected specimens (5, 7, 26, 32, 34, 37). The overall prevalence of microsporidiosis in 124 urban park pigeons was 29.0% (more than seven times higher than the prevalence in the present study), but only a single bird (0.8%) was infected with *E. hellem* (21). However, when the seven other *E. hellem*-coinfected pigeons in that study were considered (21), the overall prevalence of *E. hellem*-associated microsporidiosis in pigeons was 6.5% (almost two times higher than the overall prevalence in the present study). The overall prevalence of shedding of *E. hellem* spores by asymptomatic lovebirds (*Agapornis* spp.) was 25% (8). One surprising result of the present study is the lack of *E. bieneusi* infections in avian hosts, as this parasite species has recently been shown to be common in birds (4, 21, 29).

As important epidemiologically as the prevalence of avian infections with *E. hellem* and *E. intestinalis* is the concentration of spores in fecal pellets of birds. It is generally thought that the number of spores produced by an infected host is very high, and the infectious dose is assumed (based on animal data) to be very low (17). In AIDS patients, the concentrations of spores varied from 4.5×10^5 to 4.4×10^8 spores per ml of stool (total in 24 h, 10^{11} spores) (17). The concentration of microsporidian spores in the present study varied from 2.0×10^3 to 5.1×10^5 spores per g of feces, and the mean value for waterfowl was 3.6×10^5 spores/g. Such high concentrations of spores in fecal droppings indicate that there was indigenous infection rather that mechanical passage of spores ingested by birds from the environment.

Despite the advances in molecular technology, most epidemiological aspects of human microsporidiosis, particularly the transmission cycles, have not been resolved (11, 40, 41). Zoonotic transmission (transmission as a result of direct-contact exposure) and food-borne transmission are generally postulated to be the predominant modes of transmission (11, 25). The results of the present study demonstrate that waterborne transmission of microsporidian spores (*E. hellem*) is quite likely since predominantly waterfowl were found to carry microsporidans. The prevalence of microsporidiosis was statistically higher in waterfowl, and the aquatic status of an avian host was a predisposing factor for microsporidian infection. These findings are epidemiologically important, as most of the world's waterfowl species (i) are protected by environmental laws and occur in large numbers, (ii) have unlimited access to surface waters (including waters used for production of drinking water), and (iii) usually migrate quite long distances; in addition, most of the daily activity of these birds involves grazing in shallow waters, and the birds defecate into the water (24). It has been established by epidemiologists and the drinking water industry that the presence of aquatic birds in source water reservoirs is associated with declining water quality (1, 9, 18, 24). The present study demonstrated that very common species of waterfowl, such as mallard ducks, geese, and mute swans, can be a source of microsporidian spores of species that are known to infect humans, which are excreted into the water in large numbers.

The fecal input of aquatic birds into surface water was recently characterized by a field data-based model that predicted the impact of waterfowl visitation on water quality parameters (24). For quantitative assessment, the model considered the number of fecal pellets deposited on a 100-m-long and 1-m-wide shoreline by a single visitation of an average flock of ducks, geese, or gulls, which was 1,700 fecal pellets (24). Thus, considering that the average waterfowl fecal pellet weighs 17.2 g (18) and the finding in the present study that 8.6% of aquatic birds shed on average 3.6×10^5 spores/g, a single visitation of waterfowl can introduce into the water approximately 9.1×10^8 microsporidian spores of species known to infect humans. When the spores reach the water, several characteristics of microsporidians favor waterborne transmission (16). Under environmental conditions, spores remain infective for a long time (more than 1 year at low temperatures) (16), and because the spores are very small, water filtering is not effective for removing them (27).

Human microsporidiosis is a serious disease, which occurs more frequently now than in the past in immunocompetent and immunosuppressed people (11), and the most common species identified are *E. intestinalis*, *E. cuniculi*, *E. hellem*, and

E. bieneusi (6, 23, 41). Besides frequent intestinal infections, extraintestinal infections (e.g., urinary, respiratory, and disseminated systemic infections, sinusitis, otitis, and keratoconjunctivitis) are common (6, 13, 30, 36, 40, 41). *E. hellem*, the predominant species recovered in the present study, is particularly important clinically as it frequently produces both intestinal and extraintestinal infections, which are insidiously nonsymptomatic at the beginning (31).

As demonstrated in the present study, the sensitivity and specificity of FISH analysis of fecal samples for microsporidian spores were similar to the sensitivity and specificity of conventional staining (Chromotrope-2R and calcofluor white M2R staining). However, identification of the species of microsporidian spores is essential for pharmacological therapy as different species require different treatments. This is also important because a large number of microsporidian species are found in nonhuman hosts, including insects and fish, and the multiplicity of species complicates tracking of these parasites in environmental samples. As demonstrated here and in other studies in which the workers investigated environmentally recovered microsporidian spores infectious to humans (19, 20, 22), species of microsporidian spores can be differentiated by the multiplex FISH assay. This is particularly important because a large number of microsporidian species are found in insects, fish, and other nonhuman hosts.

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