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Quantitative Evaluation of the Impact of Bather Density on Levels of Human-Virulent Microsporidian Spores in Recreational Water▽

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Microsporidial gastroenteritis, a serious disease of immunocompromised people, can have a waterborne etiology. During summer months, samples of recreational bathing waters were tested weekly for human-virulent microsporidian spores and water quality parameters in association with high and low bather numbers during weekends and weekdays, respectively. *Enterocytozoon bieneusi* spores were detected in 59% of weekend ($n = 27$) and 30% of weekday ($n = 33$) samples, and *Encephalitozoon intestinalis* spores were concomitant in a single weekend sample; the overall prevalence was 43%. The numbers of bathers, water turbidity levels, prevalences of spore-positive samples, and concentrations of spores were significantly higher for weekend than for weekday samples; *P* values were <0.001, <0.04, <0.03, and <0.04, respectively. Water turbidity and the concentration of waterborne spores were significantly correlated with bather density, with *P* values of <0.001 and <0.01, respectively. As all water samples were collected on days deemed acceptable for bathing by fecal bacterial standards, this study reinforces the scientific doubt about the reliability of bacterial indicators in predicting human waterborne pathogens. The study provides evidence that bathing in public waters can result in exposure to potentially viable microsporidian spores and that body contact recreation in potable water can play a role in the epidemiology of microsporidiosis. The study indicates that resuspension of bottom sediments by bathers resulted in elevated turbidity values and implies that the microbial load from both sediments and bathers can act as nonpoint sources for the contamination of recreational waters with *Enterocytozoon bieneusi* spores. Both these mechanisms can be considered for implementation in predictive models for contamination with microsporidian spores.

Microsporidia are obligate intracellular eukaryotes parasitizing a wide range of invertebrates and vertebrates with over 1,200 species, of which 14 are opportunistic human pathogens, with *Encephalitozoon intestinalis*, *Encephalitozoon hellem*, *Encephalitozoon cuniculi*, and *Enterocytozoon bieneusi* being the most common (13). Microsporidia are on the Contaminant Candidate List of the U.S. Environmental Protection Agency due to their unknown transmission routes, technologically challenging identification, the inactivation of waterborne spores (24, 46), and the difficult treatment of human infections (14). Considerable evidence indicates involvement of water in the epidemiology of microsporidiosis (9, 10, 15, 16, 18, 21, 41, 45); however, this link has not been conclusively substantiated (22). Risk factor analysis for encephalitozoonosis suggested groundwater as a source of infection (18), and a massive outbreak of microsporidiosis was epidemiologically linked to a drinking water distribution system (9). Microsporidian spores have been reported in groundwater and surface water (16, 18, 21, 41), including recreational water (10); however, one study involving long-term surveillance of stools from human immunodeficiency virus (HIV)/AIDS patients with microsporidiosis has not shown a conclusive link to recreational water use (8). Waterborne spores of human-virulent species can originate from aquatic wildlife (39).

The only federal regulation regarding pathogens in recreational waters is the Beaches Environmental Assessment and Coastal Health Act, which requires the use of fecal bacterial indicators to assess water contamination (38, 47, 48). Fecal coliforms in recreational bathing waters can originate from the resuspension of bottom sediments (2, 30, 33, 43) and from the bathers themselves (7, 11, 17, 23, 31, 44). On average, the anal fecal residue being washed off to the water by a recreational bather varies from 0.14 to 10 g (23), and a load of 6×10^6 CFU of enterococci can be shed by an average recreational bather during a 15-min immersion (17). Human pathogens can also be directly released by bathers, such as individuals with gastroenteritis, children, and elderly people, via fecal accidents (23, 31, 44). Increased incidences of gastrointestinal illnesses in swimmers have been associated with higher numbers of bathers (6, 11, 34, 44). Multiple studies reported a resuspension of sediment-bound fecal coliforms in response to the disturbance of bottom sediment and sand by bathers and also due to other factors, such as intensified surface runoff, recreational boat traffic, storms, tides, and dredging (1, 2, 30, 33, 43). Information on overland migration and waterborne transport of human-virulent microsporidan spores is limited. Such spores are small (i.e., 0.7 to 2.2 μm in length) (13, 35) and thus comparable in size with fecal coliforms; similar to fecal coliforms (33), they associate with particles while in water (24, 36). Also, surface deposition is the main removal mechanism for spores

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TABLE 1. Recreational bathing water samples tested for potentially viable human-virulent microsporidian spores and for water quality[a]

Parameter	Range (mean ± SD) for:		*P* value
	Weekend samples	Weekday samples	
Spore concn (no. of spores/liter)	0–16 (4.8 ± 0.9)	0–11 (1.8 ± 0.6)	0.04
Bather density score	2–5 (3.8 ± 1.6)	0–3 (1.6 ± 1.1)	0.001
Water turbidity (NTU)	11–88 (53.6 ± 21.1)	18–75 (39.9 ± 15.4)	0.04
Rainfall (cm)	0–6.0 (1.0 ± 2.2)	0–1.8 (0.2 ± 0.5)	NS
Tide (m)	0.17–0.67 (0.33 ± 0.18)	0.16–0.46 (0.29 ± 0.12)	NS
Water salinity (ppt)	0.2–2.1 (0.9 ± 0.8)	0.3–2.0 (0.7 ± 0.6)	NS
Water temp (°C)	26.5–32.4 (29.6 ± 2.0)	22.9–33.0 (30 ± 3.0)	NS
Dissolved O_2 (mg/liter)	4.9–7.9 (6.0 ± 0.9)	4.0–7.5 (5.7 ± 0.9)	NS
Water conductivity (μS/m)	51–412 (181 ± 146)	70–396 (165 ± 125)	NS
No. of positive samples/no. of samples tested (%)	16/27 (59)[b]	10/33 (30)[b]	0.03

[a] The multiplexed FISH method was used to test for potentially viable human-virulent microsporidian spores. NTU, nephelometric turbidity units; NS, not significant.
[b] The number of positive samples and the total number of samples tested are shown. The percentage of samples testing positive is given in parentheses.

in various porous media, including sand (5). Thus, if the spores are present in the bottom sediments or in fine sand, they can be mechanically resuspended in overlying water by bathers in addition to being shed by bathers during recreational activity. A popular recreational beach area where the impact of bather density on the level of waterborne microsporidian spores could be quantitatively investigated was selected. This recreational beach attracts a large numbers a people during the weekends and very few during the weekdays due to its distal location from the city area and its relative ecological and geographical seclusion.

MATERIALS AND METHODS

Water samples were collected during 11 consecutive summer weeks from the Hammerman beach area at Gunpowder Falls State Park located on the Gunpowder River in Chase, MD (76°22′W, 39°22′N). Three samples were collected during each of 9 weekends (mainly on Sundays) and 11 weekdays (mainly Wednesday), giving a total of 27 weekend and 33 weekday samples. The ratio of weekend-to-weekday samples was uneven because the beach was closed twice during the weekend sampling period due to rainfall-related elevated coliform counts, which prevented access to the water. All samples were collected in the early afternoon into 4-liter-capacity plastic collapsible containers pretreated with 10 ml of the eluting fluid (27), transported to the laboratory in a cooler, and stored at 4°C until analyzed. Eluting fluid was added to prevent adhesion of waterborne particles to the containers' internal surfaces (27). Quality parameters of bathing water, such as dissolved O_2 level, conductivity, salinity, and temperature, were measured using a field portable meter (YSI 85; YSI Incorporated, Yellow Springs, OH), and water turbidity was measured using a colorimeter (DR/890; Hach, Loveland, CO) (Table 1). The samples were collected and bathing water quality parameters were measured in chest-deep water. The number of bathers was counted immediately prior to water collection and assigned a density score: score 5 (over 50 bathers), score 4 (38 to 49), score 3 (25 to 37), score 2 (14 to 24), score 1 (2 to 13), or score 0 (0 to 1). Bathers were usually totally immersed in water, often including their heads. Pets were not allowed to access the recreational area; however, the park was rich in various mammalian and avian wildlife species, some of which, e.g., deer, were very abundant. The 24-h rainfall data and tide levels corresponding to the water collection dates were obtained electronically (National Climate Data Center, National Oceanic and Atmospheric Administration [http://www.ncdc.noaa.gov] and Xtide Tide Prediction Server [http://www.kayaktrips.net:81]).

In the laboratory, the samples were filtered through a 3.0-μm-pore-size, 293-mm-diameter cellulose acetate membrane (Millipore, Bedford, MA) (27), and the membranes were eluted with 50 ml of eluting fluid (27) according to method 1623 (27). The tubes with eluants were centrifuged (5,000 × *g*, 5 min), and the pellet was transferred to a 15-ml-capacity plastic tube and processed by sugar-phenol floatation (3). The top 1.5 ml was collected and placed in an Eppendorf tube, and the sugar was washed off by centrifuging two times (5,000 × *g*, 5 min) using sterile phosphate-buffered saline (PBS) (pH 7.4). The resulting pellet was stored in 100 μl of sterile PBS at 4°C. The samples were coded, and the multiplexed fluorescence in situ hybridization (FISH) assay for *E. intestinalis*, *E. hellem*, *E. cuniculi*, and *Enterocytozoon bieneusi* was carried out by using Eppendorf tubes with a total volume of 100 μl of hybridization buffer at 57°C for 3 h (28, 29, 32, 39). Positive and negative controls were prepared as described previously (28, 29, 32, 39). 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 onto five lysine-coated wells (5-mm diameter) on a Teflon-coated glass slide (Carlson Scientific, Inc., Peotone, IL) and air dried. The entire area of a well was examined with the aid of an Olympus BH2-RFL epifluorescent microscope, a dry 60× objective, and a BP450-490 exciter filter. The spores were enumerated, and the samples were uncoded.

Statistical analysis was carried out with Statistix 7.0 (Analytical Software, St. Paul, MN). Variables were tested by Wilk-Shapiro ranking plots to determine whether their distribution conformed to a normal distribution; if not, nonparametric tests were used. Differences in spore concentrations, bather density scores, and water turbidity levels between weekend and weekday samples were assessed by Wilcoxon signed rank, and the chi-square test was used to assess the differences in the fractions of positive samples (Table 1). Results were presented as means ± standard deviations for continuous variables and as numbers and percentages for categorical data (Table 1). Statistical significance was considered to be a *P* value of <0.05, and all *P* values for nonparametric tests were two-tailed.

RESULTS

Human-virulent microsporidian spores were detected in 30% of samples collected on weekdays and in 59% of weekend samples (Table 1); the overall prevalence was 43%. The spores represented *Enterocytozoon bieneusi*; *E. intestinalis* spores (7 spores/liter) were concomitant in a single weekend sample that had the highest total spore concentration value, 16 spores/liter (Table 1). The percentage of microsporidian spores that did not show a positive FISH reaction but that were still identifiable as spores was very low, not exceeding a maximum of 2% of all FISH-positive spores. *Enterocytozoon bieneusi* spores stained bright yellow, and *E. intestinalis* bright red, displaying typical morphology with more intense fluorescent stain present in the polar half of the spore. The intensity and brightness of fluorescent staining of water-recovered spores were similar to the intensity and brightness observed for positive control spores.

A total of four rainfall events were associated with sampling time points; there were two consecutive weekends with 3.3 and 6.0 cm of rainfall, respectively, and on two weekdays six weeks apart, there were 1.8 and 0.3 cm of rainfall, respectively (Table 1). These two weekends had the highest bather density score values, and the two weekdays had bather density scores of 2

and 0. The Fisher exact test showed that there was no significant association between rainfall events and positivity of water samples for microsporidian spores. Timing of the rainfall did not have apparent effects on water turbidity values or spore concentration in corresponding samples.

The numbers of bathers who encountered recreational beach water on weekends were highly significantly greater than the numbers observed on weekdays (Table 1). Corresponding water turbidity levels measured on weekends were also significantly higher than those measured on weekdays (Table 1). Overall, the water turbidity values were significantly correlated with the bather density scores (Spearman's rank correlation; $R = 0.68$, $P < 0.001$); however, they were not related to the corresponding rainfall or tide levels (Table 1). The proportion of water samples containing human-virulent microsporidian spores was significantly higher in weekend water collections than in weekday samples ($\chi^2 = 4.83$, $P < 0.03$), and the concentration of spores was significantly higher in bathing water on weekends than on weekdays (Table 1). Overall, the concentration of waterborne microsporidian spores was significantly correlated with the corresponding bather density score (Spearman's rank correlation; $R = 0.53$, $P < 0.01$); however, it was not related to the rainfall and tide levels.

DISCUSSION

Enterocytozoon bieneusi and *E. intestinalis* infect both healthy and immunocompromised people, but *Enterocytozoon bieneusi* is more frequently recognized in individuals with various immunodeficiencies (13, 49). This species was described in 1985 as an intestinal pathogen of a HIV-infected patient (12), and since then, over a thousand cases have been identified in immunocompromised persons, in whom the pathogen produces life-threatening gastroenteritis. Microsporidial gastroenteritis is a serious disease, which currently occurs more frequently than in the past in both immunocompetent and immunosuppressed people (13, 35, 49). Because microsporidia are emerging human pathogens (13, 35, 49), the 50% infectious dose and minimal infectious dose are still unknown and await a volunteer experimental challenge study. However, animal data indicate that the minimal infectious dose is very low (13, 49). In contrast, the numbers of spores secreted by an infected host are very high. Asymptomatic children shed up to 1.5×10^5 *Enterocytozoon bieneusi* spores per gram of feces (37) and, on average, 3.8×10^5/g of *Enterocytozoon bieneusi* spores were released by animals (40). In AIDS patients, the concentration of spores varied from 4.5×10^5 to 4.4×10^8 per ml of diarrheic feces, totaling 10^{11} spores in a 24-h period (25).

Since all recreational water samples were collected on days deemed acceptable for bathing by fecal bacterial indicator standards, the present study provides evidence that bathing in waters open to the public can result in exposure to potentially viable microsporidian spores. It also reinforces the common doubt that bacterial indicators, i.e., *Escherichia coli* and enterococci, are not reliable in predicting the presence of other human waterborne pathogens (4, 17, 50). Unfortunately, despite the advances in molecular epidemiology, waterborne transmission cycles of microsporidian spores are still controversial and not well understood (22). The present study indicates, however, that exposure in recreational bathing waters can play a role in the epidemiology of human microsporidiosis. This is epidemiologically important information, considering the fact that over 23% of disease outbreaks associated with recreational waters in the United States were of undetermined etiology (11). The study also supports the recommendation that, irrespective of gastroenteritis etiology, symptomatic people should be advised not to enter the water, as this can result in bather cross-infection (7).

Both microsporidian species (i.e., *E. bieneusi* and *E. intestinalis*) have also been associated with a variety of wildlife mammals and aquatic birds (39, 40). Thus, the zoonotic origin of waterborne spores identified in the present study is plausible, since wildlife was abundant in the surrounding park, and spores could enter the water via surface runoff of fecal material.

Although *Enterocytozoon bieneusi* spores were frequently reported from surface waters, including recreational water (9, 10, 16, 21, 41), the testing of stools from HIV/AIDS patients with microsporidiosis has not suggested a link to recreational water use (8). However, because HIV/AIDS individuals are advised about (and are usually aware of) opportunistic pathogens in untreated surface waters, they most likely avoid such exposure, which may explain why this risk factor was not significant in the previous analysis (8).

The negative health effects of bathing in recreational waters impacted by point sources of fecal contamination is no longer a scientific controversy (11, 20, 50); currently, the bathers themselves are considered to be a nonpoint source of fecal coliforms (17). In the light of limited information on the origin and transport of waterborne microsporidian spores, the present study provides evidence that the resuspension of bottom sediments caused by bathers and the bathers' potential microbial load input to the recreational water may be considered nonpoint sources for contamination with *Enterocytozoon bieneusi* spores. However, whether the sediment resuspension or the bather microbial input played a more important role in elevated spore levels is not determined. Usually, both mechanisms are responsible for the elevated fecal coliform counts in bathing waters (7, 11, 17, 23, 31, 44), and investigation of these mechanisms' impacts on microsporidian pores requires further research.

Federal law requires the use of fecal bacterial indicators (i.e., *E. coli* and enterococci) to assess contamination of recreational bathing waters (38, 47, 48), but there are no requirements for testing such waters for other human pathogens. The recovery of waterborne microsporidian spores remains a technologically complex process, but species identification and assessment of spore viability are even more challenging. The multiplexed FISH method meets both these challenges (28, 29, 32, 39). The FISH assay employs fluorescently labeled 19-bp oligonucleotide probes targeted to *Microspora* species-specific sequences of 16S rRNA (28, 29, 32, 39). Because rRNA is present in numerous copies only in viable cells, FISH allows for species-specific identification of human-virulent, potentially viable spores (32). This is particularly important because a large number of microsporidian species are found in insects, fish, and other aquatic nonhuman hosts (26, 35). The low fraction of spores (i.e., <2%) not showing a positive FISH reaction in the present study could represent either nonviable human-virulent spores or human nonvirulent spore species; irrespectively,

these spores were not of public health importance. Unfortunately, the number of *Enterocytozoon bieneusi* spores was too low to experimentally infect immunosuppressed rodents (19) and ultimately confirm their infectivity. As demonstrated in the present work and in other studies (28, 29, 39), the simultaneous identification of environmentally recovered human-virulent microsporidian spores can be accomplished by FISH assay, which offers great benefits in comparison with other testing methods (28, 29, 39) if implemented in recreational water surveillance.

Water recreation is estimated to contribute over 85% of all United States tourist revenue (38), and the contamination of recreational waters, in addition to presenting a public health risk, threatens the revenue of recreational regions economically dependent on the public perception of them as excellent vacation destinations. Beach closings and advisory days issued for bathing waters continue to climb in the United States (38). In 2004, 85% of total closings of recreational bathing waters occurred due to exceeding bacterial indicator standards (38). This study indicates that it is essential to monitor recreational bathing areas when the number of bathers is high, i.e., on weekends and holidays, to ensure that testing accurately represents the exposure. As fecal bacterial indicators may not be indicative of human waterborne pathogens (4, 17, 50), alternative indicators for better representation within a wide diversity of waterborne pathogens should be researched. Means to decrease the negative impact of bathers on water quality include (i) limiting bather numbers, (ii) preventing diapered children from entering the water, (iii) advising people experiencing gastroenteritis to avoid contact with water, and (iv) recommending the use of showers prior to bathing. Some states have already made guidelines recommending visitor capacity levels to recreational bathing areas (42). Whenever possible, recreational bathing areas should be located away from and upstream of point-sources of contamination, such as wastewater treatment plant effluents and intensified agricultural runoff. From the recreational and drinking water perspective, future predictive models for contamination with microsporidian spores need to incorporate (i) the impact of bather density, (ii) bather microbial load into the water, and (iii) resuspension of bottom sediments.

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