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ORIGINAL PAPER

Relationships among bather density, levels of human waterborne pathogens, and fecal coliform counts in marine recreational beach water

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Abstract During summer months, samples of marine beach water were tested weekly for human waterborne pathogens in association with high and low bather numbers during weekends and weekdays, respectively. The numbers of bathers on weekends were significantly higher than on weekdays ($P<0.001$), and this was associated with a significant ($P<0.04$) increase in water turbidity. The proportion of water samples containing *Cryptosporidium parvum*, *Giardia duodenalis*, and *Enterocytozoon bieneusi* was significantly higher ($P<0.03$) on weekends than on weekdays, and significantly ($P<0.01$) correlated with enterococci counts. The concentration of all three waterborne pathogens was significantly correlated with bather density ($P<0.01$). The study demonstrated that: (a) human pathogens were present in beach water on days deemed acceptable for bathing according to fecal bacterial standards; (b) enterococci count was a good indicator for the presence of *Cryptosporidium*, *Giardia*, and microsporidian spores in recreational marine beach water; (c) water should be tested for enterococci during times when bather numbers are high; (d) resuspension of bottom sediments by bathers caused elevated levels of enterococci and waterborne parasites, thus bathers themselves can create a non-point source for water contamination; and (e) exposure to recreational bathing waters can play a role in epidemiology of microsporidiosis. In order to protect public health, it is recommended to: (a) prevent diapered children from entering beach water; (b) introduce bather number limits to recreational areas; (c) advise people with gastroenteritis to avoid bathing; and (d) use showers prior to and after bathing.

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Introduction

Cryptosporidium parvum, *Giardia duodenalis*, and human-virulent microsporidia such as *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *Encephalitozoon hellem*, and *Encephalitozoon cuniculi* are opportunistic pathogens (Graczyk et al. 2007a). These pathogens inflict considerable morbidity on healthy people and mortality in immunosuppressed individuals (Graczyk et al. 2007a). *Cryptosporidium* and *Giardia* are frequently transmitted via water, which is also involved in the epidemiology of microsporidia (Graczyk et al. 2007a).

The only federal regulation regarding testing recreational beach waters is the Beaches Environmental Assessment and Coastal Health (BEACH) Act (USEPA 2002; Wade et al. 2006). BEACH requires the testing for fecal coliforms, i.e., enterococci or *Escherichia coli*, with the frequency of one to two times per week. The U.S. EPA criteria for bathing recreational marine waters (full body contact) allows enterococci levels, i.e., geometric mean, not exceeding 35 most probable number (MPN)/100 ml, and no sample should exceed 75% of the geometric mean upper confidence limit (USEPA 2003). Fecal coliforms in recreational bathing waters can originate from re-suspension of bottom sediments (An et al. 2002; Grimes 1975; Jin et al. 2004; Steets and Holden 2003) and from bathers themselves (Cheung et al. 1991; Craun et al. 2005; Elmir et al. 2007; Gerba 2000; Steward et al. 2002); crowded beach waters usually have high fecal coliform levels (Elmir et al. 2007; Kay et al. 1994). Multiple studies reported a re-suspension of sediment-bound fecal coliforms in response to the disturbance of bottom sediments and sand by bathers, surface runoff, recreational boat traffic, storms, tides, and dredging (Ackerman and Wisberg 2003; An et al. 2002; Grimes 1975; Jin et al. 2004; Steets and Holden 2003). Human pathogens can also be directly released by bathers via fecal accidents by individuals with gastroenteritis, diapered children, and elderly people (Gerba 2000; Hanes and Fosa 1970; Steward et al. 2002). On average, the anal fecal residue being washed off to the water by a recreational bather varies from 0.14 to 10 g (Gerba 2000), and a load of 6×10^6 enterococci colony formation units (CFU) can be shed by an average bather during a 15-min-lasting immersion (Elmir et al. 2007). Recreational gastrointestinal illnesses have been positively associated with high numbers of bathers (Calderon et al. 1991; Craun et al. 2005; Johnson et al. 2008; Kay et al. 1994; Steward et al. 2002).

Recent studies demonstrated positive relationships between bather density and the levels of *Cryptosporidium*, *Giardia*, and human-virulent microsporidia in beach waters (Graczyk et al. 2007b, c; Sunderland et al. 2007). These studies (Graczyk et al. 2007b, c; Sunderland et al. 2007) indicate that the re-suspension of bottom sediments by bathers can increase the level of both fecal coliforms and waterborne parasites. The purpose of the present study was to determine relationships among bather density, levels of human waterborne pathogens, and enterococci counts in marine recreational beach water utilizing molecular techniques and a new fast enterococci test kit approved by the U. S. EPA named ENTEROLERT. A popular recreational beach area was selected where the impact of bather density on the levels of waterborne parasites and their fecal coliform indicators could be quantitatively investigated. This recreational beach attracts several hundreds people during the weekends and very few during the weekdays due to its distal location from the city and its relative ecological and geographical seclusion.

Materials and methods

Water samples were collected during 11 consecutive summer weeks over the months of July, August, and September from the Chesapeake Bay recreational beach in Maryland, USA (76°22′ W; 39°22′ N). The beach was a sandy open beach, approximately 100 m wide and 50 m long, and there were trees around. Three 4-l samples were collected at the same time during each of nine weekends and 11 weekdays giving a total of 27 weekend and 33 weekday samples (Table 1). Three samples, evenly spaced along the beach width, were taken at the chest-deep water into collapsible plastic containers. The number of weekend to weekday samples was uneven because the beach was closed for two weekends due to the elevated enterococci levels. Water quality parameters such as temperature, dissolved O_2, salinity, and conductivity were measured using a portable meter YSI Model #85 (YSI Incorporated, Yellow Springs, OH, USA), and water turbidity was determined by a portable DR/890 Colorimeter (Hach, Loveland, CO, USA) (Table 1). The numbers of bathers were counted and assigned a density score as described previously (Graczyk et al. 2007b) (Table 1). The 24-h rainfall data and tide levels corresponding to the water collection dates were obtained electronically (NRDC 2005) (Table 1). Enterococci levels were: (a) retrieved electronically for the corresponding collection dates from the local Department of Environmental Protection and Resource Management (DEPRM) website; and (b) determined using the ENTEROLERT test kit (Idexx Laboratories Inc., Westbrook, ME, USA). The local DEPRM utilized standard U.S. EPA method 1600 (USEPA 1998, 2000, 2001) for determination of enterococci level in marine beach waters; only six weekend and eight weekday enterococci values were posted electronically, and enterococci levels were presented in MPN/100 ml (Table 1). The local DEPRM

Table 1 The results of testing of recreational marine bathing water samples for viable *Cryptosporidium parvum* oocysts, *Giardia lamblia* cysts, and *Enterocytozoon bieneusi* spores by the multiplexed fluorescence in situ hybridization (FISH) method, and for water quality parameters

	Weekend samples n=27	Weekday samples n=33	P value
Number and (%) of *C. parvum*-positive samples	13 (48)	2 (6)	0.02
Number and (%) of *G. lamblia*-positive samples	10 (37)	2 (6)	0.02
Number and (%) of *E. bieneusi*-positive samples	16 (59)	10 (30)	0.03
Range and mean±SD of *C. parvum* concentration (oocyst/l)	2–42, 13.7±1.7	0–7, 1.5±0.2	0.01
Range and mean±SD of *G. lamblia* concentration (cysts/l)	0–33, 9.1±1.1	0–4, (0.6±0.1)	0.01
Range and mean±SD of *E. bieneusi* concentration (spores/l)	0–16, 4.8±0.9	0–11, 1.8±0.6	0.04
Range (geometric mean) of enterococci level			
ENTEROLERT test kit (MPN/100 ml)	5–55 (38)	5–11 (7)	0.001
Health Department Data[a] (MPN/100 ml)	10–50, (28)	10–700, (175)	0.001
Range and mean±SD of bather density score	2–5, 3.8±1.6	0–3, 1.6±1.1	0.001
Range and mean±SD of water turbidity (NTU)	11–88, 53.6±21.1	18–75, 39.9±15.4	0.04
Range and mean±SD of rainfall (cm)	0–6.0, 1.0±2.2	0–1.8, 0.2±0.5	NS
Range and mean±SD of tide (m)	0.17–0.67, 0.33±0.18	0.16–0.46, 0.29±0.12	NS
Range and mean±SD of water salinity (ppt)	0.2–2.1, 0.9±0.8	0.3–2.0, 0.7±0.6	NS
Range and mean±SD of water temperature (°C)	26.5–32.4, 29.6±2.0	22.9–33.0, 30±3.0	NS
Range and mean±SD of dissolved O_2 (mg/l)	4.9–7.9, 6.0±0.9	4.0–7.5, 5.7±0.9	NS
Range and mean±SD of water conductivity (μS/m)	51–412, 181±146	70–396, 165±125	NS

[a] Based on six weekend and eight weekday samples

collected the water samples from sites closely located to our sampling sites.

For analysis of waterborne parasites, water samples were filtered through a 1.2-μm-pore-size, 293-cm-diameter cellulose acetate membrane (Millipore, Bedford, MA, USA), and the membranes were eluted with 50 ml of eluting fluid (Graczyk et al. 1997). The tubes with eluants were centrifuged (5,000×*g*; 5 min), and the pellets were transferred to15-ml plastic tubes and processed by sugar–phenol flotation (Graczyk et al. 2007a; Kahler and Thurston-Enriquez 2007). The top 1.5 ml was collected, placed in an Eppendorf tube, and the sugar was washed off by centrifugation two times (5,000×*g*; 5 min) using sterile phosphate buffered saline (pH7.4). The samples were coded and the multiplexed fluorescence in situ hybridization (FISH) assay combined with immunofluorescent antibody staining was carried out for *C. parvum* and *G. duodenalis*, and multiplex FISH was carried out for *E. intestinalis*, *E. hellem*, *E. cuniculi*, and *E. bieneusi* (Graczyk et al. 2007a). The reported efficiency of the method is 78% (Graczyk et al. 1997). Positive and negative controls were prepared as described previously (Graczyk et al. 2007a). Viable pathogens were enumerated (Graczyk et al. 2007a), and the samples were uncoded.

To confirm the identification of *C. parvum*, samples were analyzed utilizing an SSU rRNA-based nested polymerase chain reaction–restricted fragment length polymorphism (PCR–RFLP) with restriction enzymes SspI and VspI as described previously (Jiang et al. 2005; Xiao et al. 2006). Nucleic acid for PCR analyses was obtained with an aid of proteinase K. Briefly, each sample was assayed using 2 μl of the DNA template per PCR mixture. Residual PCR inhibitors in the extracted DNA were neutralized by adding 400 ng/μl nonacetylated bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA). Ten microliters of the secondary PCR products was digested at 37°C overnight in a 40-μl reaction mixture. The digested products were visualized by 2% agarose gel electrophoresis.

To confirm the identification of microsporidian spore species, spore-positive samples were assayed by PCR using primers based on the 18Sr RNA gene for detection of *E. intestinalis*, *E. hellem*, *E. cuniculi*, and *E. bieneusi* (daSilva et al. 1996, 1997, 1999; deGroote et al. 1995; Visvesvara et al. 1995). Briefly, 0.3 μM concentrations of each primer and AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA) were mixed in a 50-μl final volume. The cycling parameters were 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 1 min, with a final extension of 72°C for 7 min. Negative controls and positive controls with the spores of *E. intestinalis*, *E. hellem*, *E. cuniculi*, and *E. bieneusi* were included in the PCR testing. All PCR products were analyzed on a 2% agarose gel (Agarose GTG/LE; American

Bioanalytical, Natick, MA, USA) and stained with ethidium bromide for visualization.

Statistical analysis was carried out with Statistix 7.0 (Analytical Software, St. Paul, MN, USA). Variables were tested by Wilk–Shapiro/ranking plots to determine whether their distribution conformed to a normal distribution, and if not, non-parametric tests were used. Differences in pathogen concentrations, bather density score, and water turbidity 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). Associations between variables were tested by Pearson correlation coefficient. Results were presented as mean±SD for continuous variables and as number and percentage for categorical data (Table 1). Statistical significance was considered to be $P<0.05$, and all P values for non-parametric tests were two-tailed.

Results

FISH assays identified *C. parvum* oocysts, *G. duodenalis* cysts, and *E. bieneusi* spores in water samples. Most pathogens detected by the FISH assays were viable as per FISH analysis; a fraction of non-viable cells was approximately less than 2% of all parasites. *Cryptosporidium parvum* was the only species identified by PCR–RFLP, and PCR confirmed the presence of *E. bieneusi* DNA.

The numbers of bathers on weekends were significantly greater ($P<0.001$) than on weekdays, and water turbidity was also significantly higher ($P<0.04$) on weekends than on weekdays (Table 1). Overall, the water turbidity values were significantly correlated with the bather density score (Pearson correlation coefficient; $R=0.68$, $P<0.001$); however, they were not related to the rainfall or tide levels, indicating that bathers themselves caused re-suspension of sediments and increase of water turbidity values. The Fisher exact test showed that there was no significant association ($P>0.101$) between rainfall levels and: (a) positivity of water samples for *C. parvum* oocysts, *G. duodenalis* cysts, and *E. bieneusi* spores; (b) enterococci levels; and (c) water turbidity values.

The proportion of water samples containing *C. parvum* oocysts, *G. duodenalis* cysts, and *E. bieneusi* spores was significantly higher ($P<0.03$) in weekend than in weekday water collections, and the concentrations of all these pathogens was significantly higher ($P<0.04$) on weekends when compared to weekdays (Table 1). Enterococci levels determined by the ENTEROLERT test kit were significantly higher ($P<0.001$) in weekend samples than in weekdays; however, this relationship was reversed for enterococci data obtained from the local DEPRM website (Table 1). Overall, the concentration of all three species of waterborne parasites was significantly correlated with bather density score (Pearson correlation coefficient; $R=0.63$, $P<0.01$). There was a significant temporal correlation between enterococci levels determined using the ENTEROLERT test kit and concentration of *C. parvum* oocysts (Pearson correlation coefficient; $R=0.95$, $P<0.01$), *G. duodenalis* cysts (Pearson correlation coefficient; $R=0.01$; $P=0.018$), and *E. bieneusi* spores (Pearson correlation coefficient; $R=0.66$, $P=0.04$).

Two of six (33%) and six of eight (75%) enterococci level values posted by the local DEPRM website for weekend and weekday timepoints, respectively, exceeded the allowable U.S. EPA limit of 35 MPN/100 ml. Using the ENTEROLERT test kit, 18 of 27 (67%) enterococci weekend readings exceeded the U.S. EPA allowable limits, and by contrast none of weekday reading exceeded U.S. EPA allowable limits.

Discussion

In terms of microbiological quality of recreational bathing water, bathers themselves sustain a non-point source of fecal coliform bacteria (Elmir et al. 2007); however, it is still not clear whether sediment re-suspension or direct bather microbial input plays a more important role in water contamination. Usually both mechanisms are suggested to be responsible for elevated enterococci counts in surface recreational waters (Cheung et al. 1991; Elmir et al. 2007; Gerba 2000; Steward et al. 2002). The present study supported previous findings that elevated enterococci levels were positively associated with intensified attendance of beachgoers to recreational water (Conteas et al. 1998; Elmir et al. 2007; Gerba 2000; Steward et al. 2002). The current study also indicates that the re-suspension of bottom sediments by bathers caused elevated levels of waterborne parasites and enterococci as it is unlikely that the bather direct input could cause simultaneous elevation of all three waterborne parasites, i.e., *C. parvum*, *G. lamblia*, *E. bieneusi*, together with the enterococci levels.

Irrespective of a causative mechanism for elevated levels of waterborne parasites and their fecal indicators, the concentrations of *C. parvum* oocysts, *G. duodenalis* cysts, and *E. bieneusi* spores showed (particularly *C. parvum* and *G. duodenalis*) significant correlation with enterococci readings determined by the ENTEROLERT test kit. Fecal coliforms received a lot of negative publicity, being generally criticized as not reliable predictors for protozoan and viral pathogens in drinking, reclaimed, recreational, and surface waters (Elmir et al. 2007). Significant correlation between *Giardia* cysts and enterococci and *E. coli* counts was reported previously (Coupe et al. 2006), and enterococci were found to be a good predictor of gastrointestinal illnesses after swimming in contaminated

waters (Wade et al. 2006). The present study provides evidence that enterococci levels could be a good indicator for *Cryptosporidium*, *Giardia*, and microsporidian spores specifically for recreational beach waters as the same mechanism, i.e., re-suspension of bottom sediments caused elevated levels of waterborne protozoan parasites and enterococci. Because this is the first study to evaluate relationships among bather density, waterborne parasites, and their fecal coliform indicators, additional studies will be required to evaluate and generalize these findings. ENTEROLERT test kit offers a serious advantage for testing of bathing water as the results can be provided next day as opposed to longer time required by the standard U.S. EPA method 1600 (USEPA 2003).

According to these criteria for enterococci levels in bathing recreational marine waters (35 MPN/100 ml), our enterococci data showed that this beach should be closed on six of nine (67%) weekends; however, it could remain open during all weekday timepoints. The present study, together with previous studies (Graczyk et al. 2007b, c; Sunderland et al. 2007), indicates that it is essential to monitor recreational bathing areas during times when the number of bathers is high, i.e., on weekends and holidays, to ensure that testing accurately represents the exposure to waterborne pathogens. Beach closings threaten the revenue of recreational regions which are economically dependent on the public perception of them as excellent vacation destinations. Water recreation is estimated to contribute over 85% of all U.S. tourist revenue (NOAA 2007) and, therefore, favors microbiological testing of bathing waters by local authorities on days that would yield the lowest counts of fecal bacterial indicators in order to keep the recreational areas open.

Despite enterococci readings exceeding U.S. EPA criteria of 35 MPN/100 ml posted on three separate weekday timepoints (i.e., 250 and 750 MPN/100 ml, 220 and 120 MPN/100 ml, and 40 and 50 MPN/100 ml, respectively) by the local authorities responsible for bacteriological monitoring of this beach, the beach remained open to the public on these days. Thus, the present study provides direct evidence that bathing in waters open to the public can result in exposure to human parasites. The high enterococci levels observed on weekdays could be due to the runoff or precipitation.

The study supports the recommendation that people with gastroenteritis should not enter the water as this can result in bather cross-infection (Cheung et al. 1991). Means to decrease the negative impact of high levels of bathers on recreational water quality include: (a) limiting bather numbers; (b) preventing diapered children from entering the water; (c) advising people with gastroenteritis to avoid bathing; and (d) recommending the use of showers prior to bathing. Also, whenever possible, recreational bathing areas should be located away and upstream of point sources of contamination (Steward et al. 2002). The need for improved public health and protection from gastrointestinal diseases at beaches was addressed through the passage of the BEACH Act in 2000 (NRDC 2005; USEPA 2003; Wade et al. 2006); however, it does little to protect the public from waterborne protozoa and viruses in recreational waters. To address this risk, the California Department of Health Services has issued guidelines recommending visitor capacity numbers for water recreation per beach area (Standish-Lee and Loboschefsky 2006).

E. bieneusi spores were previously reported from recreational waters (Coupe et al. 2006); however, epidemiological analysis of HIV/AIDS patients with microsporidiosis did not suggest association with recreational water (Conteas et al. 1998). As HIV/AIDS individuals are usually aware of opportunistic pathogens in untreated surface waters, they avoid such exposure, which explains why this risk factor was not significant in this analysis (Conteas et al. 1998). Because microsporidia are emerging pathogens, the ID_{50} or minimal infectious doses are still unknown. Unfortunately, despite the advances in molecular epidemiology, waterborne transmission cycles of microsporidian spores are still controversial or not well understood (Franzen and Muller 1999). The present study indicates that exposure to recreational bathing waters can play a role in the epidemiology of human microsporidiosis, which is an important finding considering the fact that over 23% of disease outbreaks associated with recreational waters in the USA are due to undetermined etiology (Craun et al. 2005).

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