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Occurrence of *Cryptosporidium*, *Giardia*, and *Cyclospora* in influent and effluent water at wastewater treatment plants in Arizona

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HIGHLIGHTS

- Occurrence of three genera of protozoa in wastewater was determined over a year.
- *Giardia* was always abundant than *Cryptosporidium* in influent wastewater.
- The identified *Giardia intestinalis* strains belonged to two assemblages, AII and B.
- *Cyclospora cayetanensis* was detected from wastewater using a newly developed qPCR.
- Efficacy of the conventional wastewater treatment at removing (oo)cysts is limited.

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ABSTRACT

We investigated the occurrence of *Cryptosporidium*, *Giardia*, and *Cyclospora* at two wastewater treatment plants (WWTPs) in Arizona over a 12-month period, from August 2011 to July 2012. Influent and effluent wastewater samples were collected monthly, and protozoan (oo)cysts were concentrated using an electronegative filter, followed by the detection of protozoa using fluorescent microscopy (*Cryptosporidium* oocysts and *Giardia* cysts) and PCR-based methods (*Cryptosporidium* spp., *Giardia intestinalis*, and *Cyclospora cayetanensis*). The concentration of *Giardia* cysts in the influent was always higher than that of *Cryptosporidium* oocysts (mean concentration of $4.8–6.4 \times 10^3$ versus $7.4 \times 10^1–1.0 \times 10^2$ (oo)cysts/l) with no clear seasonality, and $\log_{10}$ reduction of *Giardia* cysts was significantly higher than that of *Cryptosporidium* oocysts for both WWTPs ($P < 0.05$). Log_{10} reduction of *Giardia* cysts at the WWTP utilizing activated sludge was significantly higher than the other WWTP using trickling filter ($P = 0.014$), while no statistically significant difference between the two WWTPs was observed for the $\log_{10}$ reduction of *Cryptosporidium* oocysts ($P = 0.207$). Phylogenetic analysis revealed that *G. intestinalis* strains identified in wastewater belonged to two assemblages, AII and B, which are potentially infectious to humans. *C. cayetanensis* was also detected from both influent and effluent using a newly developed quantitative PCR, with the highest influent concentration of 1.2×10^4 copies/l. Our results demonstrated that these protozoan pathogens are prevalent in the study area and that efficacy of the conventional wastewater treatment processes at physically removing (oo)cysts is limited.

1. Introduction

Cryptosporidium and *Giardia* are the most common waterborne protozoan pathogens in industrialized countries (Karanis et al., 2007; Baldursson and Karanis, 2011). They infect domestic and wild animals, which may shed a large number of (oo)cysts in the environments (Plutzer et al., 2010; Smith and Nichols, 2010). Waterborne *Cryptosporidium* and *Giardia* infections are particularly important because their transmissive stages, i.e., oocysts and cysts, respectively, are highly stable in the environments and mostly resistant to disinfectants (such as chlorine and chloramines) (Carey et al., 2004; Erickson and Ortega, 2006; Plutzer et al., 2010), which makes it difficult to control their risks to humans via the exposure to contaminated water. In fact,

Abbreviations: WWTP, wastewater treatment plant; EPA, Environmental Protection Agency; GDH, glutamate dehydrogenase; PBS, phosphate buffered saline; IMS, immunomagnetic separation; IFA, immunofluorescent assay; ITS, internal transcribed spacer; qPCR, quantitative polymerase chain reaction; RT, reverse transcription; RFLP, restriction fragment length polymorphism.

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numerous waterborne outbreaks of giardiasis and cryptosporidiosis have been documented all over the world (Karanis et al., 2007; Baldursson and Karanis, 2011), which has led the U.S. Environmental Protection Agency (EPA) to regulate the levels of *Cryptosporidium* oocysts and *Giardia* cysts allowed in drinking water (U. S. Environmental Protection Agency, 2005).

Cyclospora cayetanensis is an intestinal coccidian pathogen that is transmitted by environmentally resistant oocysts through ingestion of contaminated food and water (Ortega and Sanchez, 2010). *C. cayetanensis* can cause prolonged diarrhea, nausea, abdominal cramps, and other significant adverse health effects in humans (Ooi et al., 1995; Wurtz et al. 1993; Ortega and Sanchez, 2010). Humans appear to be the only natural host for this parasite (Eberhard et al., 2000; Chacín-Bonilla, 2010). *C. cayetanensis* is endemic in developing regions, such as Haiti, Guatemala, Peru, and Nepal, while in developed countries *C. cayetanensis* infection is mainly identified in travelers from areas of endemicity (Chacín-Bonilla, 2010; Ortega and Sanchez, 2010). However, recent large *Cyclospora* outbreaks in multiple states in the U.S. associated mainly with fresh produce among people who had no history of international travel during the 14 days prior to onset of illness indicate the incidence of food- and waterborne *Cyclospora* infections within the U.S. (Centers for Disease Control and Prevention, 2013a); it was indicated that contaminated imported fresh produce contributed to the outbreaks (Centers for Disease Control and Prevention, 2013b). Although there are reports that actually suggest waterborne transmission of *C. cayetanensis* (Rabold et al., 1994; Aksoy et al., 2007; Huang et al., 2013), information on the occurrence and fate of *C. cayetanensis* in the environment is limited compared with that of *Cryptosporidium* and *Giardia*. This is partly because a standardized detection protocol, such as the USEPA Method 1623 for *Cryptosporidium* and *Giardia* (U. S. Environmental Protection Agency, 2005), has not yet been established for *C. cayetanensis*.

In the present study, we applied novel detection tools for *Cryptosporidium*, *Giardia*, and *Cyclospora* to detect these protozoa in the influent and treated effluent of two WWTPs located in southern Arizona monthly over a one-year period. Protozoan (oo)cysts were concentrated by a method using an electronegative microporous membrane, followed by the detection of protozoa using fluorescent microscopy and PCR-based methods. The detected *Giardia intestinalis* strains were characterized based on the glutamate dehydrogenase (GDH) gene sequences to determine their genetic diversity in wastewater and possible source of excretion.

2. Materials and methods

2.1. Collection of wastewater samples

Between August 2011 and July 2012, influent and effluent wastewater grab samples were collected monthly from two WWTPs located in southern Arizona. Plant A utilized a conventional activated sludge process and Plant B utilized a biological trickling filter process or "biotower". In addition, both plants used chlorination for disinfection. The characteristics of each plant are described in Table 1. All samples were collected in sterile plastic bottles, stored on ice, and transported to the laboratory, where they were processed within 12 h of collection.

2.2. Concentration of protozoa in wastewater samples

A total of 48 wastewater samples (12 influent and 12 effluent samples from each of the two plants) were collected, and protozoa in the samples were concentrated using an electronegative filter method, which is capable of concentrating viruses as well as protozoa in water samples, as described previously (Haramoto et al., 2012), with slight modifications. Briefly, the wastewater samples (100 ml influent and 1000 ml effluent) were passed through the electronegative filter (cat. no. HAWP-090-00; Merck Millipore, Billerica, MA) attached to a glass filter holder (Advantec, Tokyo, Japan), followed by an acid rinse and elution of viruses from the filter (Katayama et al., 2002). To recover the protozoan (oo)cysts retained on the filter after the elution of viruses, the filter was detached from the glass filter holder, aseptically cut in half, and one half of the filter was vigorously vortexed in the presence of a ball-shaped stirring bar and 10 ml of an elution buffer containing 0.2 g/l $Na_4P_2O_7 \cdot 10H_2O$ (Kanto Chemical, Tokyo, Japan), 0.3 g/l EDTA($C_{10}H_{13}N_2O_8$)$\cdot 3Na \cdot 3H_2O$ (Wako Pure Chemical Industries, Osaka, Japan), and 0.1 ml/l Tween 80 (Research Organics, Cleveland, OH) in a 50-ml plastic tube. The water portion of the sample was recovered in another 50-ml plastic tube. The same procedure was repeated twice with 10 and 5 ml of the elution buffer, and approximately 25 ml of the resulting protozoa-concentrated sample was obtained.

2.3. Immunomagnetic separation

The 25-ml protozoa-concentrated sample was centrifuged at 2000 ×*g* for 10 min at 4 °C, the supernatant was carefully removed, and the pellet was suspended with 10 ml of phosphate buffered saline (PBS). The tube was centrifuged again at 2000 ×*g* for 10 min at 4 °C and the resulting pellet was suspended in 10 ml of PBS. To purify *Cryptosporidium* oocysts and *Giardia* cysts, the sample was subjected to immunomagnetic separation (IMS) using the Dynabeads GC-Combo (Life Technologies, Carlsbad, CA) following the manufacturer's protocol with slight modifications. In brief, Dynabeads® anti-*Cryptosporidium* and Dynabeads® anti-*Giardia* were added to the sample, followed by a rotation for 1 hour at room temperature. Subsequently, the Dynabeads-organism complexes were pelleted using a Dynabeads® MPC®-1 magnet, and the supernatant (approx. 10 ml) was recovered for the *Cyclospora* assay. To wash the Dynabeads-organism complexes, the pellet was resuspended in 10 ml of PBS. The Dynabeads-organism complexes were pelleted again using the Dynabeads® MPC®-1 magnet, and the supernatant (approx. 10 ml) was also recovered for the *Cyclospora* assay, which was combined with the previously obtained supernatant and resulted in obtaining a total of approx. 20 ml sample for the *Cyclospora* assay.

Cryptosporidium oocysts and *Giardia* cysts were acid-dissociated from the Dynabeads according to the manufacturer's protocol with slight modification. Briefly, 50 μl of 0.1 N HCl (Kanto Chemical) was added to the tube and the pellet was resuspended by vigorous vortexing. The Dynabeads were pelleted again using the Dynabeads® MPC®-S magnet, and the supernatant (50 μl) was collected in a tube containing 10 μl of 1.0 N NaOH for neutralization. This (oo)cyst dissociation procedure was repeated once more using 50 μl of 0.1 N HCl and the supernatant was recovered in the same tube to obtain purified (oo)cysts

Table 1
Characteristics of WWTPs studied.

Plant	Biological treatment process	Service population	Treatment capacity (m^3/day)	Water quality characteristics of effluent[a]		
				BOD (mg/l)	TSS (mg/l)	*E. coli* (MPN/100 ml)
A	Activated sludge	Approx. 5.0×10^5	1.55×10^5	11–28	4–26	<1–134
B	Trickling filter	Approx. 2.5×10^5	9.46×10^4	8–27	9–25	7–225

[a] Values observed during the study period, August 2011 to July 2012. BOD, biochemical oxygen demand; TSS, total suspended solid; MPN, most probable number.