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Prevalence of *Cystoisospora belli* in Wastewater Treatment Plants in Sharkeya, Egypt

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Abstract: Discharge of untreated or insufficient treated wastewater may result in water borne *Cystoisospora belli* outbreaks. Influent and effluent wastewater samples from two different wastewater treatment plants (WWTPs) were collected monthly for the detection of *Cystoisospora belli* using centrifugal concentration and purification by zinc sulphate floatation, followed by molecular detection using real-time PCR. Results showed that the prevalence rates of *Cystoisospora belli* in influents and effluents of WWTPs were 14.5% and 6.2%, respectively. The removal rates of *Cystoisospora belli* oocysts in WWTP (A) utilizing activated sludge and WWTP (B) using trickling filter were 66.7% and 50%, respectively. There was no significant difference between removal of *Cystoisospora belli* in WWTP (A) and WWTP (B). The prevalence rates of *Cystoisospora belli* oocysts in raw and finally treated wastewater of WWTP (A) were 12.5% and 4.2%, respectively, while their prevalence rates in raw and treated wastewater of WWTP (B) were 16.7% and 8.3%, respectively. In conclusion, further studies are needed to clear the actual prevalence of *Cystoisospora belli* in the environment.

Key words: *Cystoisospora belli*, wastewater treatment plants, real-time PCR.

Introduction

The world's population is increasing and concentrating in urban centres. This trend is particularly strong in developing countries, as additional 2.1 billion people are expected to be living in cities by 2030 (Hindiyeh, 2004; American Water Works Association. 2006). These cities produce wastes (sludge and wastewater) in billions of tons yearly. Different fates of these wastes are categorized depending on the locality of the cities: whether wastes are regularly collected or not, submitted to treatment or not and finally used directly or indirectly without beneficial use (American Water Works Association 2006; Hindiyeh, 2004). Globally, wastewater discharges are incriminated in carrying human pathogens, such as enteric protozoa into surface waters. When a leakage occurred in wastewater treatment process, parasitic stages can easily reach final effluents that may cause possible outbreak of diseases. If the final effluents are not sufficiently treated before being discharged into a nearby water body, the corresponding treatment plants are considered a potential source of contamination to our watersheds (Hindiyeh, 2004; Lim et al., 2007).

Cystoisospora, formerly known as *Isospora*, is one of the protozoan parasites that cause a disease known as cystoisosporiasis in enteric tract of humans and animals (Frenkel et al., 1979; Atambay et al., 2007). *Cystoisospora belli* (*C. belli*) is a human parasite associated with severe and chronic diarrhea, in particular, for AIDS and other immunocompromised individuals and persons living with them (Cimerman

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et al., 1999; Lewthwaite et al., 2005; Atambay et al., 2007). This parasite infected children and travellers to tropical regions (Okhuysen, 2001; Bialek et al., 2002; Jongwutiwes et al., 2007). *Cystoisospora* can be acquired through the ingestion of sporulated oocysts in water or food (Ortega and Cama, 2018). Although symptoms in immunocompetent individuals are self-limiting, early diagnosis and treatment can minimize the period of symptoms considerably. Microscopic examination of direct smear for the detection of *Cystoisospora* oocysts reveals thin transparent shell of oocysts, which is somewhat difficult. To improve detection sensitivity, additional concentration, staining methods and microscopic examination were performed (Franzen et al., 1996; Lainson and da Silva, 1999; Bialek et al., 2002).

Recently, real-time PCR proved to be a sensitive and specific alternative technique for the diagnosis of intestinal protozoa parasites (Braun-Kiewnick and Kiewnick, 2018). Till now, there is gap of knowledge about the prevalence of *C. belli* and its removal in different wastewater treatment plants. Therefore, in the present study, we aimed to assess the prevalence and the removal percentages of *C. belli* in different wastewater treatment plants by real-time PCR.

Material and Methods

Sampling Sites and Sampling

A total of 96 wastewater samples were collected from two biological wastewater treatment plants (A and B WWTPs) with different secondary treatment technologies located in Sharkeya governorate, Egypt. The examined WWTP (A) used activated sludge as a secondary treatment technology, while WWTP (B) used trickling filter technology. Sampling sites for each wastewater treatment plant were influent and final effluent. Each sampling site was regularly sampled twice per month for one year period during 2016.

The operational system of the WWTP (A) is composed of coarse screens, the FOG removal chambers (to remove fat, oil and grease), primary sedimentation basin, aeration basin, secondary sedimentation basin and lastly effluent disinfection step with 0.5 to 1 ppm chlorine to destroy most of pathogenic microbes. WWTP (A) produces 10,000 cubic metres of treated wastewater per day.

WWTP (B) is composed of bar-screen to remove large objects as sticks, cans and debris which may cause flow obstructions. This is followed by FOG removal chambers, primary sedimentation basin and trickling filter in which an attached-growth biological process that uses an inert medium to attract microorganisms, secondary sedimentation basin in which microorganisms and other solids are settled and the final step of disinfection with chlorine 0.5 to 1 ppm as a free chlorine final concentration.

Cystoisospora belli Concentration and Purification

Samples (1 litre volume each) were separately collected in clean polypropylene plastic containers and sent to the laboratory at the same day of collection. Wastewater samples were centrifuged at 4000 g at 4 °C for 30 min. The supernatant was discarded and the remaining pellet was subjected to flotation technique to collect the oocysts out of the surrounding debris using zinc sulphate ($ZnSO_4$) solution (specific gravity 1.3) according to Moodley et al. (2008). After floatation, the obtained pellet was re-suspended in PBS (pH 7.4) and subsequently centrifuged for 15 min at 4300 g at 4 °C, and suspended in 200 µl AL buffer (Qiagen, Hilden, Germany) (Berglund et al., 2017).

Nucleic Acid Extraction

Nucleic acids were extracted from 200 µl of the final concentrated suspension using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Three freezing-thawing cycles (each cycle consisted of 2 min in liquid nitrogen followed by 2 min in boiling water) were performed before the application of the manufacturer's protocol. The obtained DNA was stored at −20 °C until used.

Nucleic Acid Amplification

Cystoisospora belli-specific primers Ib-40F (5′-ATATTCCCTGCAGCA TGTCTGTTT-3′) and Ib-129R (5′-CCACACGCGTATTCCAGAGA-3′) were selected to amplify a fragment of 89bp (Ten Hove et al., 2008). Real-time PCR was done using 10 µl Sso Advanced universal SYBR green Super mix (BIO-RAD, USA), 3 µl template DNA, 2 µl of both forward and reverse primers in a total volume of 20 µl. The real-time PCR amplification thermal conditions were as follows: 3 min initial denaturation step at 95 °C, 40 cycles of denaturation at 95 °C for 15 s and annealing-extension at 58 °C for 30 s. The specificity of the reactions was determined by melting curve analysis of the amplicons.

Statistical Analysis

The obtained data were analyzed using Paired *t* test in Minitab statistical program (Minitab Inc., Pennsylvania, USA). *P* values less than 0.05 were considered significant (Wild, 2005).

Results

The overall prevalence rates of *C. belli* in influents and effluents of the examined WWTPs were 14.5% and 4.2%, respectively. Total removal rate of *C. belli* oocysts for both WWTPs was 57.1%. The removal rates of *C. belli* oocysts in WWTP (A) and WWTP (B) were 66.7% and 50%, respectively. There was no significant removal of *C. belli* in WWTP (A) (*P* = 0.166) and WWTP (B) (*P* = 0.166). The prevalence rates of *C. belli* oocysts in raw sewage and final treated effluents of WWTP (A) were 12.5% and 4.2%, respectively. Consequently, the prevalence of *C. belli* oocysts in raw sewage and treated effluents of WWTP (B) were 16.7% and 8.3%, respectively (Table 1, Figure 1).

Table 1: Prevalence of *Cystoisospora belli* in influent and effluent of wastewater treatment plants

WWTPs		*Cystoisospora belli*	
		No	*%*
Activated sludge (WWTP A) (*n* = 48)	Influent	3	12.5
	Effluent	1	4.2
	Removal	2	66.7
	P	0.166	
Trickling filter (WWTP B) (*n* = 48)	Influent	4	16.7
	Effluent	2	8.3
	Removal	2	50
	P	0.166	
Total (*n* = 96)	Influent	7	14.5
	Effluent	3	6.2
	Removal	4	57.1

Seasonally, *Cystoisospora belli* DNA were only observed in spring and summer in percentages 33.3%

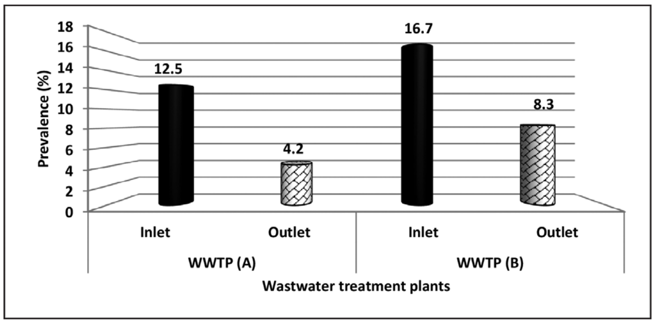

Figure 1: Prevalence of *Cystoisospora belli* in inlets and outlets of wastewater treatment plants.

and 16.7%, respectively in inlet samples of WWTP (A), while no oocysts of *C. belli* were noticed in winter and autumn. Concerning WWTP (B), the prevalence of *C. belli* was similar in each of spring and summer (33.3% for each), while no oocysts were detected in winter and autumn (Table 2).

Table 2: Seasonal variations of *Cystoisospora belli* in inlets of examined wastewater treatment plants

Seasons	*Total collected samples*	*Inlet samples*			
		WWTP (A)		*WWTP (B)*	
		No.	*%*	*No.*	*%*
Winter	6	0	0	0	0
Spring	6	2	33.3	2	33.3
Summer	6	1	16.7	2	33.3
Autumn	6	0	0	0	0

Discussion

Wastewater treatment consists of physical (filtration and sedimentation), biological (trickling filters, stabilization ponds and activated sludge), and chemical processes (ozone, chlorine, and chlorine dioxide disinfection) (Hindiyeh, 2004; Drechsel et al., 2015). Data about *Cystoisospora belli* in the environment are much more limited worldwide and in Egypt are nearly absent. Results of the present study showed that the prevalence rates of *Cystoisospora belli* in influents and effluents of the examined WWTPs were 14.5% and 4.2%, respectively. Out of 325 reported water-associated protozoan disease outbreaks worldwide, 0.9% was caused by *Cystoisospora belli* (APHA, 2012). In Dakahlia Governorate, Egypt, the occurrence of *Isospora belli* in potable water samples reached 0.47% (Elshazly et al., 2007). Microbial pathogens including viruses, bacteria, protozoa and helminths are considered risk factors especially for farm workers and their families working in fields irrigated with contaminated wastewater (Drechsel et al., 2015). In Egypt, *Isospora* oocysts were seen in 4% of the feces of goats (Elmadawy and Diab, 2017). Detailed knowledge of the epidemiology of *Isospora* infection is lacking, including reservoirs, risk places, and risk times. Person-to-person transmission is probably rare (Mirdha et al., 1993).

Isospora has commonly been found in AIDS patients with diarrhea in Brazil (9.9%), Zaire (12%), Zambia (16%), and Haiti (12%) (Cimerman et al., 1999). These rates are much higher than the recorded data from developed countries, suggesting that travelling to the tropics may increase risk, even for healthy travellers. *Isospora* was detected in people with HIV from several countries including USA (0.8% and 3.6% and 5.4%) (Sorvillo et al., 1995; Mathewson et al., 1998), Italy (0.6%) (Brandonisio et al., 1999), France (0.2% and 0.4%) (Guiguet et al., 2007; Lagrange-Xelot et al., 2008), Senegal (15.3%) (Dieng et al., 1994), Ethiopia (1.6%) (Fisseha et al., 1999), Guinea-Bissau (10.8%) (Lebbad et al., 2001), Nigeria (7.5%) (Keshinro et al., 2003), Thailand (5%) (Wiwanitkit et al., 2001), India (20%) (Shah et al., 2016), Cuba (1.5%) (Escobedo et al., 1999), Mexico (0.5%) (Moran et al., 2005), Brazil (6.8%) (Assis et al., 2013) and Iran (2.5%) (Salehi Sangani et al., 2016). Immunocompetent persons are also infected with *I. belli* (Cimerman et al., 1999). Unfortunately, there was no data available about the removal of *Cystoisospora belli* oocysts in different wastewater treatment plants. In the present investigation, the removal rates of *Cystoisospora belli* oocysts in WWTP (A) and WWTP (B) were 66.7% and 50%, respectively. Other researchers declared that conventional domestic wastewater treatment processes may not be totally effective for inactivating parasites (Reimers et al., 1981).The expected removal of protozoa cysts in activated sludge wastewater treatment process was from 0-1 $\log_{10}$ (Hindiyeh, 2004). No statistical significance for the removal of *Cryptosporidium* oocysts (P = 0.207) was observed between WWTP utilizing activated sludge and that using trickling filters, although $\log_{10}$ reduction of *Giardia* cysts at the WWTP utilizing activated sludge was significantly higher than the other WWTP using trickling filter (P = 0.014) (Kitajima et al., 2014).

In conclusion, statistically there was no considerable difference between the two examined WWTPs for the removal of *Cystoisospora* oocysts, although the activated sludge treatment system showed better removal results than trickling filters. Further studies are needed to clear the actual prevalence of *Cystoisospora belli* in the environment.

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