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Genotypic detection and evaluation of the removal efficiency of *Giardia duodenalis* at municipal wastewater treatment plants in Northern South Africa

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Abstract. Over the past decade, *Giardia duodenalis* has increasingly been implicated in diarrheal outbreaks and water and wastewater have been recognized as important vehicles for diseases. Although studies have reported the occurrence of these parasites in developed countries, their occurrence in water and wastewater bodies in these countries including South Africa has not been thoroughly investigated. In the present study, wastewater samples from 6 different sewage treatment plants in the Vhembe District were collected for a period of 12 months. The samples were concentrated and tested for the presence of *G. duodenalis* using both microscopy and polymerase chain reaction methods targeting the *tpi* gene. Of the 79 wastewater samples tested, 25 (31.65%) were positive. Of these, 15 (60%) were assemblage A, while 8 (32%) were assemblage B and 2 samples (8%) were positive for both genogroups. Assemblage A was more common in February 2010 while assemblage B showed two peaks in December-January and March-April and was not detected in May 2010. The general removal rate was 40% for plants using biological filters and 20% for plants using activated sludge. The present study has shown that *Giardia* assemblage A is more common in sewage treatment plants in the Vhembe District, but the removal efficiency was low. This represents a public health hazard since these organisms might contaminate drinking water sources. Therefore action needs to be taken for the design of more effective procedures or methods for the removal of these parasites from the environment in order to avoid potential outbreaks.

INTRODUCTION

Giardia is a flagellated protozoan which infects a wide range of vertebrate hosts. The genus currently comprises six species (i.e., *Giardia agilis*, *Giardia ardeae*, *Giardia duodenalis*, *Giardia microti*, *Giardia muris* and *Giardia psittaci*), which are distinguished on the basis of the morphology and ultrastructure of the trophozoite (Adam, 2001). *Giardia duodenalis* (syn. *G. intestinalis*, *G. lamblia*) is the only species found in humans. It is also found in other mammals, including pets and livestock (Thompson *et al.*, 2008). This protozoan produces robust cysts, which are voided in the faeces and transmitted directly through faecal/oral contact, or by ingesting contaminated water or food. Giardiasis is a widespread intestinal disease, which is believed to be responsible for 2.5 million diarrhoea associated deaths and nutritional deficiencies in children in developing countries (Ignatius *et al.*, 2012). However, very few studies have been conducted on this parasite in South Africa and in the Limpopo Province in particular.

Over the past decade, water has been increasingly recognized as an important vehicle for diseases, and many waterborne diseases outbreaks have been reported in economically developed countries such as the USA, the United Kingdom and Japan (Smith *et al.*, 2006; Baldursson & Karanis,

2011; Serdarevic *et al.*, 2012). Organisms of the *Giardia* genus can be assigned to at least seven distinct assemblages (A–G) based on genetic analyses (Monis *et al.*, 2003). Only assemblages A and B have been detected in humans, as well as in a wide range of other mammals. The organism has been found in more than 40 animal species (Meyer, 1994). Waterborne outbreaks of giardiasis have been reported for a period spanning 30 years (Craun, 1990). In the USA for example, *Giardia* is the most commonly identified pathogen in outbreak investigations, with more than 100 waterborne outbreaks, based on epidemiological evidence (Daly *et al.*, 2010). Waterborne outbreaks have also been reported in Australia, Canada, New Zealand, Sweden, and the United Kingdom (Karanis *et al.*, 2007). These outbreaks have been linked to consumption of untreated surface water contaminated by human sewage, as well as to groundwater that was contaminated by human sewage or contaminated surface water (Chalmers *et al.*, 2010; Tien & Earn, 2010). Therefore it is important to monitor the occurrence of these organisms in environmental sources in order to provide measures that would curb the occurrence of diarrheal outbreaks in human communities.

In South Africa, most wastewater treatment plant effluents are directed to the rivers or are used for different purposes such as irrigation of fruits and vegetables (Gumbo *et al.*, 2010). This constitutes a significant risk of infections of the populations through water, food or direct contact with the treated wastewater that might not be properly disinfected. Studies conducted in a different region of South Africa indicated the occurrence of *Giardia* in wastewater samples (Dungeni & Momba, 2010). However, the distribution of *Giardia* as well as the efficiency of wastewater treatment plants in the removal of these organisms from the wastewater in the Northern region of the South Africa is not known. Furthermore the genotypic distribution of *Giardia* in wastewater and their differential removal from wastewater is not known. Therefore, the present study investigated the occurrence of *G. duodenalis* from wastewater and treated wastewater from municipal sewage treatment plants in the Vhembe district, Limpopo Province and used PCR methodology for the determination of the genotypes in association with the pH and turbidity. The removal efficiency of the plants was also evaluated over a period of 12 months.

MATERIAL AND METHODS

Sample collection

At the beginning of the study, the Department of Water Affairs in the Vhembe District was contacted for preliminary arrangements. The authority was informed about the project and permission was obtained to visit the sewage treatment plants for sample collection. The municipality was also informed of the project and the plant operators were informed as well. Once the support of the authorities was obtained the plants were visited for sample collection. The samples were collected from all the six different sewage treatment plants in the Vhembe District, Limpopo province and this included Vuwani, Elim, Louis Trichardt, Malamulele, Tswinga and Watervaal. The samples were collected in 5 litre bottles, once a month in August 2009, December 2009, January 2010 to July 2010. At least three samples were collected from each treatment plant including the final effluent, wastewater from the secondary tanks and the influent (Raw sewage). Once collected, the samples were transported immediately to the Microbiology laboratory at the University of Venda for analysis. In the laboratory, the pH of the samples was measured as well as the turbidity. The processes used by the different sewage treatment plants were activated sludge, biological filtration and ponds (Table 1).

Sample processing

The samples were processed using three different methods including centrifugation, ferric sulphate flocculation and cesium chloride flocculation. A set of 12 samples were used in order to compare the three methods for the concentration of cysts in the sewage samples.

Table 1. Information on the sewage treatment plants where samples were collected

Name of the Plant	Treatment processes	Size (in Mega litres)	Type and origin of waste	Average population served
Vuwani STP	Activated sludge, Chlorination	2 ML	Home based waste	<10000
Elim STP	Activated sludge, Maturation ponds, Chlorination	2–5 ML	Hospital based waste	<10000
Louis Trichardt STP	Biological filters, Anaerobic digesters, Maturation ponds, Chlorination	10–20 ML	Home and Industrial waste	20000 – 50000
Malamulele STP	Biological digesters, Anaerobic digesters, Maturation ponds, Chlorination	5–10 ML	Home based waste	10000 – 20000
Tswinga STP	Chemical pre-treatment, Primary tanks, Biological filters, Anaerobic digesters, Maturation ponds, Chlorination	10–20 ML	Home based waste	50000 – 100000
Watervaal STP	Activated sludge, maturation ponds, Chlorination	2–5 ML	Home based waste	10000 – 20000

Centrifugation method

Wastewater samples were analyzed using centrifugation method. Briefly, for each sample 150 ml were distributed in ten 15 ml conical tubes. The tubes were then centrifuged at 1500 rpm for 20 minutes. After centrifugation the supernatant was discarded and all the deposits were collected into one tube. This was repeated three times for each sample collected in order to process a total of about 500ml of the sewage sample. The tubes with deposits were then stored at -20ºC until further analysis.

Concentration of water samples by flocculation

Different flocculation methods have been described and evaluated. These include flocculation by Ferric sulphate, aluminium sulphate and calcium carbonate. The evaluation of these three methods by Karanis & Kimura (2002) indicated that ferric sulphate had low impact on oocysts and cysts viability and high recovery rates and therefore was considered a useful method for the detection of cysts and oocysts in environmental water samples. Therefore, this method was used for the rest of analysis. A total of 25L of each sample were treated with 50 ml of an aqueous Ferric sulphate solution to give a final concentration of 16mg/ml and the pH was adjusted to 6 and mixed by regular shaking for 5 min. The samples were left overnight in the dark at room temperature, to allow flock to precipitate. Supernatants were discarded the next day by the use of a vacuum pump and about 200 ml-pellets was transferred into 50-ml polypropylene tubes and centrifuged at 1500 rpm for 20 min at room temperature. The supernatant was discarded and the pellets from the same sample (4 x1-mL pellets) were united and centrifuged again. After discarding the supernatant, a portion of the resulting pellets (1 mL) was re-suspended in 1mL lysis buffer [8.4 g citric acid monohydrate, 17.64 g trisodium citrate dihydrate, distilled water up to 100 mL; pH 4.7]. After settlement with the lysis buffer for 1 h at room temperature (vortexing every 15 min), the samples were washed twice with distilled water and a final pellet was obtained and kept in the freezer until further analysis.

Sample Analysis

DNA purification

Two hundred and fifty milligram (250 mg) of the concentrate was frozen at -20ºC for 30 min and then was thawed in boiling water for 5 min for a total of seven freeze-thaw cycles. The genomic DNA was then purified

from the suspension using the QIAamp DNA Stool Mini Kit from Qiagen (Valencia, CA, USA) following the manufacturer's instructions.

Detection of *Giardia duodenalis* from the wastewater concentrates

The sewage samples concentrates were subjected to six cycles of freezing-thawing (-80ºC/+ 80ºC, 30 min) and incubated with Proteinase K (1 mg/ml) and lysis buffer (NaCl 0.15 M, EDTA 0.1 M, SDS 0.5%, pH 7-8) at 37ºC for 24 h (Polverino *et al.*, 2004). This suspension was stored at -20ºC until DNA isolation. Genomic DNA was purified from the cysts by the use of the QIAamp DNA stool Mini kit, (Qiagen, Valencia, CA, USA) as described above. A PCR-RFLP previously described by Minvielle *et al.* (2008) with modifications. Briefly, 3mM MgCl2, 0.1 microgram/microlitre bovine serum albumin and 0.1 U/microlitre Taq DNA polymerase (Invitrogen). All reactions involved an initial denaturation step at 94ºC (4 min), 30 cycles at 94ºC (30 s), 52ºC (30 s) and 72ºC (1 min), with a final elongation step at 72ºC (10 min). A BioRad thermocycler was used. Both positive and negative controls were included in each PCR to validate results. Amplification was assessed by electrophoresis of PCR samples in 1.5% or 3.2% agarose gels, depending on the expected sizes of amplified DNA.

Statistical analysis

All the data collected were uploaded onto an excel sheet. The Statistical package for Social Sciences (SPSS) program, Version 10.0 was used for analysis. The statistical analysis was performed through the chi-square test and the differences were considered significant when the P value was less than 0.05.

RESULTS

General occurrence of *Giardia* in wastewater in the region

Prior to the study, three methods were evaluated for the concentration of cysts in the sewage samples. This set of experiments indicated that the flocculation by ferric sulphate yielded much better results. Therefore, ferric sulphate flocculation was used for the rest of analysis. *Giardia duodenalis* was detected from the wastewater concentrates by PCR after DNA purification. Both assemblages A and B were detected since these are the two assemblages affecting humans. A total of 79 wastewater samples were tested for *Giardia* by PCR. Of these, 25 (31.65%) were positive. Of the 25 samples that were positive for *Giardia*, 15 (60%) were assemblage A, 8 (32%) were assemblage B while 2 samples (8%) were positive for both assemblages. *Giardia* was more common in samples from Watervaal occurring in 50% of the samples tested, followed by Malamulele with 41.7% of the samples that tested positive. The general distribution of *Giardia* in the sewage treatment plants is shown in Table 2. *Giardia* occurred most commonly in August 2009 compared to all the other months and occurred less in March 2010. *Giardia* occurred more often in February 2010 and was detected in five plants out of the 6 plants tested.

Effect of pH and turbidity on the occurrence of *Giardia* in the wastewater samples

Sixty four samples with known pH were tested for *Giardia* by PCR. Of these 2 had a pH less than 6 and none of them was positive for *Giardia*. Fifty four had a pH varying from 6 to 8.5 and of these 15 were positive. Four samples had a pH more than 8.5 and of these samples, 2 were positive for *Giardia*. *Giardia* occurred mostly in samples with a turbidity level between 11 and 50 NTU while a few samples with turbidity above 51 were positive for *Giardia*. Figure 1 shows the occurrence of *Giardia* at different turbidity levels in the wastewater samples.

Efficiency of the sewage treatment plants to eliminate /reduce *Giardia* from the wastewater

The general efficiency of the plants to reduce *Giardia* from the wastewater was determined by comparing the occurrence in the influent to that of the final effluent. It was established

Table 2. Removal efficiency of *Giardia duodenalis* by the sewage treatment plants

		Treatment process		Total
		Biological filter	Activated sludge	
Giardia (total)	INFLUENT	5 (39%)	5 (33%)	10 (36%)
	2nd TANK	4 (31%)	4 (27%)	8 (29%)
	FINAL	3 (23%)	4 (40%)	7 (30%)
Removal efficiency		40%	20%	30%
Giardia assemblage B	INFLUENT	4 (80%)	0	4 (40%)
	2nd TANK	4 (100%)	1 (25%)	5 (63%)
	FINAL	1 (33%)	0	1 (14%)
Removal efficiency		75%	0%	75%
Giardia assemblage A	INFLUENT	2 (40%)	5 (100%)	7 (70%)
	2nd TANK	0	4 (100%)	4 (50%)
	FINAL	2 (67%)	4 (100%)	3 (86%)
Removal efficiency		0%	0%	0%

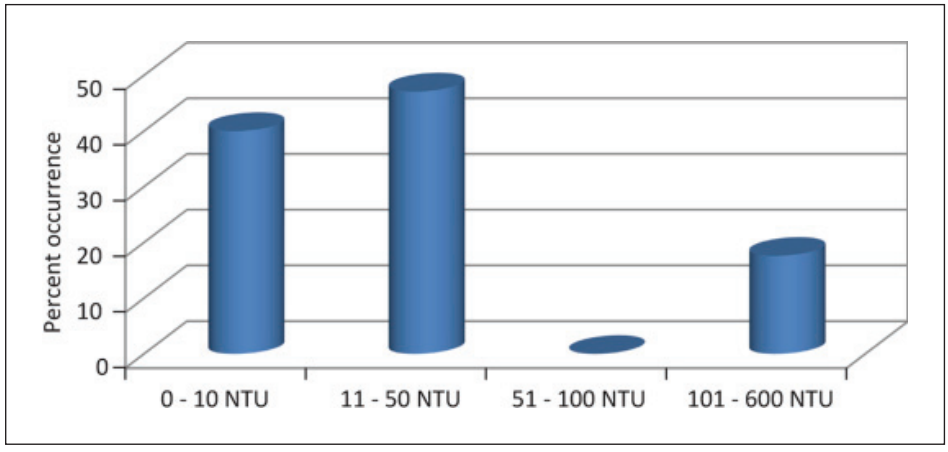

Figure 1. Occurrence of *Giardia* in the wastewater samples at different turbidity levels

that the elimination efficiency was only 15.4% of the *Giardia* that come in the plants. The percentages of occurrence of *Giardia* in the different points of the sewage treatment plants are shown in Figure 2. Of all the plants, there were some that had more *Giardia* in the effluent than in the influent namely Elim and Malamulele while others did not have *Giardia* in the final effluent such as Louis Trichardt and Tswinga. The number of times *Giardia* was found in the influent was the same as when it was found in the effluent indicating that there was no elimination of the organisms in the wastewater following treatment.

Effect of the main treatment processes on the occurrence of *Giardia* at different points of the sewage treatment in the plants

The impact of the main treatment processes on the elimination of *Giardia* was evaluated. Globally plants that used biological filters reduced more *Giardia* than the plants using activated sludge. The general reduction rate was 40% for biological filters and 20% for activated sludge. Assemblage A occurred mostly in plants with activated sludge while assemblage B occurred mostly in the plants with biological filters. There was a high

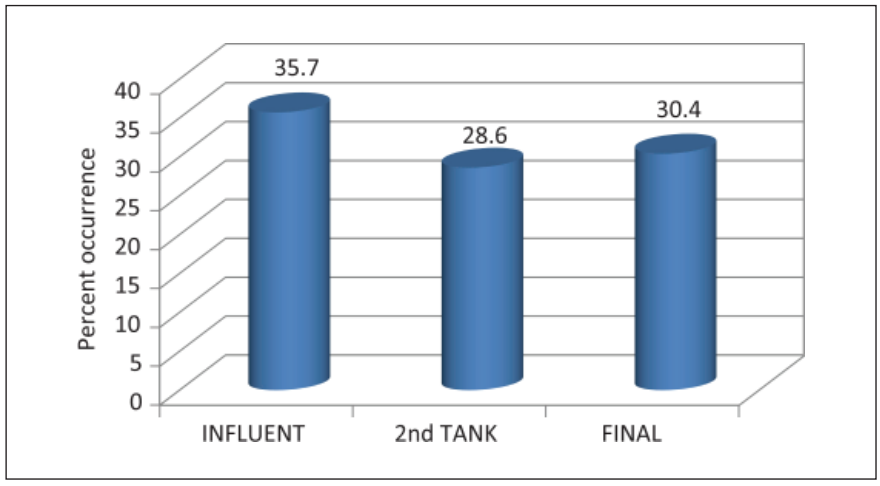

Figure 2. Occurrence of *Giardia* at different levels in the sewage treatment plants

Table 3. Occurrence of the different *Giardia* assemblages in the Sewage treatment according to treatment processes

Organisms	Treatment process		Total
	Biological filter	Activated sludge	
Giardia PCR	12 (31%)	13 (33%)	25 (32%)
Giardia assemblage B*	9 (75%)	1 (8%)	10 (40%)
Giardia assemblage A**	4 (33%)	13 (100%)	17 (68%)
Total	12	13	25

* For assemblage B: χ^2=11.779; p=0.001
** For assemblage A: χ^2=12.745; p<0.001

rate of reduction of assemblage A by the biological filters while the reduction of assemblage B was generally poor for both the activated sludge plants and the biological filters plants. The results are shown in Table 3.

Distribution of *Giardia* assemblages in the wastewater samples

Of the two *Giardia* assemblages tested, assemblage A was the most common and was detected in 17 wastewater samples whereas assemblage B was detected in 10 samples. Both assemblages (mixed infections) occurred only in the same sample at the same time on two different occasions, one in Tswinga in the influent, and one in Watervaal in the sedimentation tank but no mixed infections were detected in the final effluent. Only assemblage A was detected in Elim and Vuwani, while only assemblage B occurred in Louis Trichardt. Both assemblages were found in the other plants at different times of sample collection. There was a sense of seasonal distribution of assemblage A which was more common in February 2010 with the lowest percentage of occurrence in April and May 2010. Assemblage B showed two peaks in December-January and March-April and was not detected in May 2010 (Figure 3).

Giardia assemblage B occurred mostly in the secondary tanks and was reduced from the plant as the percentage of occurrence in the final effluents was much lower (Figure 4). Assemblage A was more persistent in the wastewater with the percent occurrence in

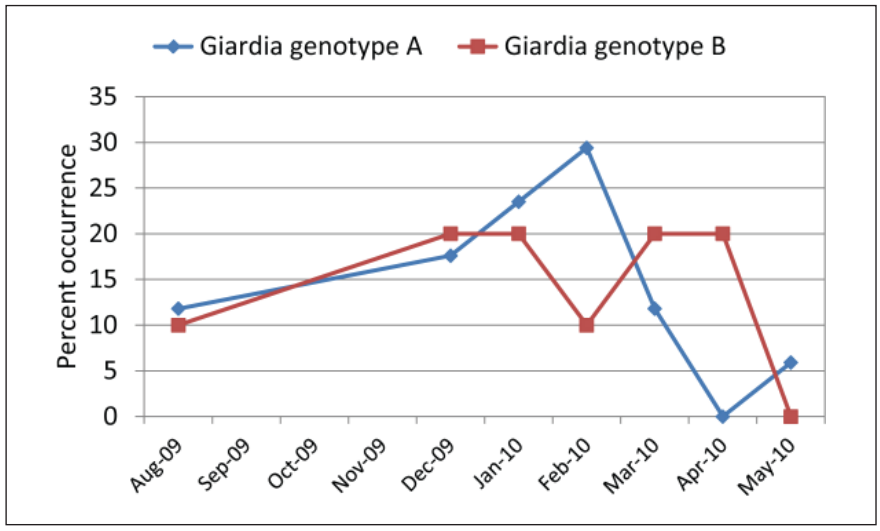

Figure 3. Occurrence of *Giardia* in wastewater and treated wastewater collected at the sewage treatment plants in the Vhembe District

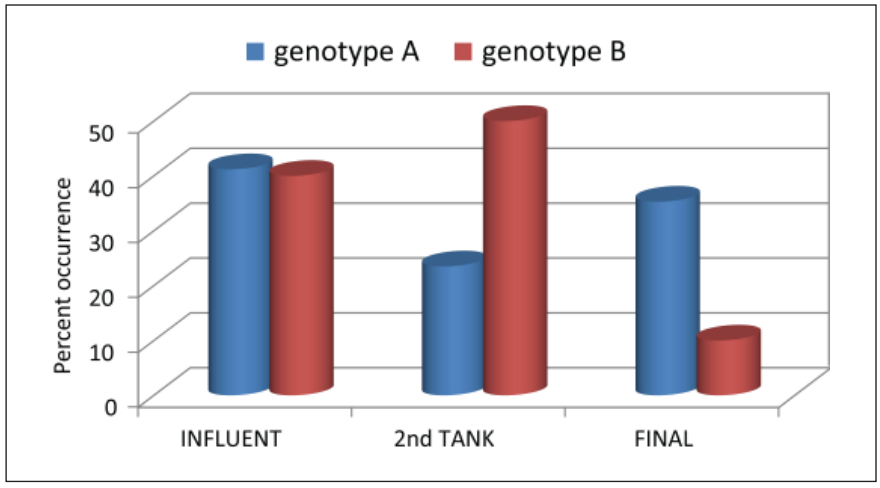

Figure 4. Occurrence of the different *Giardia* assemblages in the wastewater and treated wastewater samples

the influent close to that in the final effluent. In Malamulele, Assemblage A was not found in the influent but was detected in the final effluent while the assemblage B that was detected from the influent was not eliminated in the effluent.

Effect of treatment processes on the occurrence of *Giardia* in the sewage treatment plants

Giardia was detected from plants using both major treatment processes however, assemblage B was mainly encountered in plants with Biological filters (75%) while assemblage A was mainly encountered in plant with activated sludge. Table 3 shows the distribution of *Giardia* by treatment process.

DISCUSSION

The main objective of Sewage treatment plants is to remove pathogens as well as

chemicals from wastewater before it is sent back to nature. The treated wastewater can further be used for other purposes such as agriculture, human consumption or in industrial settings. Therefore, the efficiency of removal of pathogens in the sewage treatment plants is crucial to the establishment of a community with less pathogenic organisms and reducing the risks of infections in the community. In South Africa and particularly in the Limpopo Province, sewage treatment plants are mostly situated in urban centers while most rural communities are deprived of these sanitation infrastructures. Previous studies have demonstrated that sewage treatment in Mpumalanga Province which is close to the Limpopo Province produced final effluent that still contained potentially pathogenic bacterial organisms (Samie *et al.*, 2009). However, very limited or no study has been conducted in the sewage treatment processes in the Limpopo Province. In the present study, the 6 conventional sewage treatment plants in the Vhembe District were evaluated for the presence of *G. duodenalis* assemblages in wastewater and treated wastewater samples for a period of 12 months between August 2009 and July 2010.

Giardia is among the most common parasitic organisms transmitted by water and they have been involved in most of waterborne outbreaks of diarrhoea throughout the world. In order to elucidate the epidemiology of these parasites in particular populations or geographical regions, several authors have analyzed sewage influents for *Giardia* cysts (Robertson *et al.*, 2006a). Additionally, such analyses can be used as an indirect method of assessing the occurrence of these infections in human populations (Robertson *et al.*, 1995). This is particularly useful in communities where many members of the population are HIV infected and are therefore more susceptible to opportunistic infections. Measurements of concentrations of parasites in sewage influent over time may provide information on temporal variation in the occurrence of infections, but as flow rates also vary according to precipitation and industrial contributions, such data must be treated cautiously.

In a study in Norway, the analysis of samples of sewage influent from 40 sewage treatment works (STW) throughout the country for *G. duodenalis* cysts showed that these parasites were detected frequently. In fact, 93% of STW were *Giardia* positive with maximum concentrations of >20,000 parasites/liter (Robertson *et al.*, 2006a; Li *et al.*, 2012). In the present study, *Giardia* occurred in all STPs at different periods and was detected in 32% of all the samples tested. This prevalence is lower than those described in Norway. This could be due to the difference in the amount of samples tested or methodology used or just the geographical locations of the sampling sites. Both assemblage A and B of *Giardia* were detected in the plants of the Vhembe District with assemblage A being the most common. These results are similar to those described in Brazil where Assemblage A was found in about 80% of the samples (Fernandes *et al.*, 2011). Some studies have suggested that *Giardia* assemblage A infections may be less symptomatic than those from assemblage B (Homan *et al.*, 2001). In the present study, *Giardia* assemblage A was easily eliminated from the wastewater samples than assemblage B even though assemblage A was more common. In a study in Norway, assemblage A *Giardia* appeared to be more common and more widespread than those from assemblage B. An Italian study (Caccio *et al.*, 2003) also demonstrated that assemblage A *Giardia* cysts occurred more frequently than assemblage B in sewage samples. In a Milwaukee study (Sulaiman *et al.*, 2004), *Giardia* cysts isolated from approximately 85% of 131 wastewater samples were assemblage A, which is similar to our results. However, in the Milwaukee study, by sequencing at a single gene, over 96% of the assemblage A isolates were considered to be of identical sub assemblage. In Norway, a giardiasis outbreak described in Bergen in 2004 and 2005 was believed to have been caused by an assemblage related to B3 (Robertson *et al.*, 2006b) and was presumed to have resulted from sewage

leakage into the water supply. The persistence of *Giardia* assemblage B in the present study constitute a potential risk to the population getting infected with this assemblage and a potential outbreak caused by these organisms.

For some samples at several STPs, the number of parasites detected in the effluent was greater than in the influent sample collected on the same day. This may be an artifact of higher method recovery efficiencies with the cleaner effluent samples or a reflection of temporal fluctuations in parasite concentrations, the lack of "pairing" of the samples, the uneven distribution of parasites in the sample matrices, or a combination of these factors. Similar results have been described previously by Robertson *et al.* (2006a). Removal efficiency estimates should also be treated with caution. Failure to obtain positive PCR results from wastewater samples containing *Giardia* cysts has also been reported by others (Caccio *et al.*, 2003). It is unknown whether this was due to insufficient DNA or inhibition of the PCR.

In Italy, studies by Caccio *et al.* (2003) as well as Di Benedetto *et al.* (2005) have indicated that more *Giardia* cysts were found in raw sewage and primary effluent (up to 108,000/l) compared to *Cryptosporidium* oocysts. High concentrations of *Giardia* cysts, and to a lesser extent of *Cryptosporidium* oocysts, were also present after secondary treatment of wastewater by activated sludge and sedimentation (Lonigro *et al.*, 2006). It has been shown that tertiary treatments significantly reduce cyst and oocyst density in wastewater (Carraro *et al.*, 2000). Final disinfection with chlorine has little effect on oocyst density (Briancesco & Bonadonna, 2005; Kothavade, 2012), and chlorine and UV treatments may not influence *Giardia* cyst viability (Brandonisio *et al.*, 2007). In the STPs studied, there was provision for chlorination of the final effluent even though in some cases the chlorine was not replaced on time after it had been exhausted. This is useful for bacterial organisms and of little use for parasitic organisms. The improve-ment of sewage treatment is therefore very important for the reduction or complete elimination of these organisms in the environment as well as the associated diarrhoea outbreak risks.

Sewage effluent containing parasitic organisms can contaminate water sources. In a study of a Surface Water Treatment Plant (WTP) in Brazil, *Giardia* spp. was detected in 87.5% of the water samples analyzed with densities ranging from 2.5 to 120 cysts per L (Neto *et al.*, 2010). In this study, *Giardia* cyst concentrations detected were elevated and were associated with discharge of untreated sewage in the River. It is therefore advised that measures should be taken to protect surface water from sources of contamination. Similarly, the high occurrence rates and low removal rate of *Giardia* in wastewater in our study need urgent attention. Additional treatment of sludge is also indicated such as composting with vegetable matter that could be done by "cooking" the sludge to control parasitic infections. Criteria for sludge pasteurization as currently applied in Switzerland and Germany at 55 to 60ºC for 30 minutes may offer a considerable margin of safety and could be adopted in South Africa in order to improve the parasitological quality of the effluents and resulting sludge.

Giardia assemblage A is more common in our environment, but the removal efficiency of this strain was high. However, the removal efficiency for *Giardia* assemblage B by the sewage treatment plants was very low. Therefore there is need to design more effective procedures or methods for the removal of *Giardia* from the environment in order to avoid any potential outbreaks based on *Giardia* (especially, assemblage B) because of its accumulation in the environment. This study provides a quantitative basis for risk assessment studies and development of mitigation strategies, such as improving wastewater treatment efficiency. Further studies are needed in order to elucidate the impact of environmental contamination by these organisms on human health particularly in rural areas where people still use untreated water for everyday consumption.

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