Molecular surveillance of *Cryptosporidium* and *Giardia duodenalis* in sludge and spent filter backwash water of a water treatment plant

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ABSTRACT

The purpose of this study was to monitor the presence of *Cryptosporidium* spp. and *Giardia duodenalis* in a water treatment plant (WTP) using settling sludge and backwash water (BW) samples in previous and post the deflocculation of polyaluminium chloride (PAC) flacks. Eleven collections were performed. BW and settling sludge (SSF) were concentrated by calcium carbonate flocculation, and another aliquot of settling sludge (SSC) by centrifugation. The samples were divided as follows: Group A, containing 33 samples without degradation of PAC flakes, and Group B, with degradation by alkalinization with 10 M NaOH. Sample DNA was extracted with a commercial kit, and nested polymerase chain reaction (PCR) was used to detect *Cryptosporidium* and *G. duodenalis*. All samples from Group A were negative for *Cryptosporidium* spp., and 6.1% (2/33) were positive for *G. duodenalis* in SSC samples. While the absence of *Cryptosporidium* may be due to a low contamination level of the water resource, the presence of *G. duodenalis* indicates contamination of the raw water. The detection of *G. duodenalis* in SSC samples indicates that this detection method was the most effective. The 33 samples from Group B were negative for both protozoa, probably due to the presence of aluminium and humic substances.

Key words | flocculation, PAC, PCR, protozoan, waterborne, water treatment plant

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INTRODUCTION

Water is the primary route of transmission of pathogens associated with outbreaks and massive epidemics, with an estimated 3.5 million deaths caused by waterborne diseases (Prüss-Üstün *et al.* 2008; Tien & Earn 2010). *Cryptosporidium* spp. and *Giardia duodenalis* are parasites with zoonotic potential, and are the primary causes of waterborne outbreaks due to protozoa in the world (Baldursson & Karanis 2011). An outbreak related to backwash water (BW) recirculation in a water treatment plant (WTP) has been described (Richardson *et al.* 1991).

There is great concern about the presence of these protozoa in raw water pumped to WTPs, because *Cryptosporidium* oocysts and *Giardia* cysts are not inactivated in simplified WTPs, due primarily to resistance to chlorine-based disinfectants. The process of retention in decanters or filters is the most effective way to prevent these protozoa from reaching conventionally treated water (Maciel & Sabogal-Paz 2016). However, research on the presence of protozoa in spent filter BW and settling sludge in WTPs is scarce (Osaki *et al.* 2013). Karanis *et al.* (1996) detected *Cryptosporidium* and *Giardia* in BW of a WTP and proved the capacity of backwash reuse for contamination of raw water and risks of surface water use for health.

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Previous studies found these protozoa in water from Brazilian municipality WTPs (Almeida *et al.* 2015). In Brazil, no surveillance for the parasites is demanded so, the potential risk of drinking water transmitting these protozoa to the population are not so clear. The objective of this study was to monitor the presence of *Cryptosporidium* spp. and *G. duodenalis* in WTPs, using settling sludge and spent filter BW as samples, and previous and post deflocculation of polyaluminium chloride (PAC) flakes with three methods of concentration.

METHODS

This study was conducted in Londrina, the second largest municipality in Paraná, Brazil, with 558,439 inhabitants, and sanitation indicators of 99.98 and 88.53% for residences with tap water and treated sewage respectively (IBGE 2017). Samples were collected from a conventional-system WTP supplied by the Tibagi River which is responsible for 57% of the treated water in Londrina.

Eleven collections were performed every 15 days between June and November 2015. For sludge, 5 L of residual sludge from the settling tank were collected and for BW, 5 L of residual water from the filter backwash process were collected. The samples were transported to the Laboratory of Veterinary Protozoology of the State University of Londrina (UEL) for processing.

From each collection, three samples were subjected to different methodologies: (i) 11 spent filter BW samples were tested using flocculation and centrifugation (BW); (ii) 11 residual settling sludge samples were tested using flocculation and centrifugation (SSF); and (iii) 11 residual sludge samples were tested using centrifuge concentration (SSC); for a total of 33 samples.

Because samples were obtained post PAC flocculation in the WTP, two methodologies were used to recover (oo) cysts from settling sludge. Spent filter BW samples were concentrated by calcium carbonate flocculation using a method adapted from Greinert *et al.* (2004). We used 2,000 mL from a previously homogenized initial sample, and added 1% $NaHCO_3$ (1 M) and $CaCl_2$ (1 M). The solution was homogenized, pH was adjusted to 10 with NaOH (1 M), and the sample was decanted overnight. Supernatant was removed by vacuum aspiration, and 20% of H_3NSO_3 (10% w/v) was added to the residual solid. Residual solution was transferred to 50-mL tubes with 5% Tween 80 solution (0.1%) and centrifuged at $3{,}000 \times g$ for 10 min, then the supernatant was discarded and the centrifugation step was repeated. The pellet was stored at −20 °C.

To concentrate (oo)cysts in settling sludge, we used the flocculation technique described above and in another aliquot the centrifugal concentration method adapted from Santos *et al.* (2011). Briefly, the initial sample was homogenized, and 570 mL was uniformly divided into twelve 50 mL tubes with 5% Tween 80 solution (0.1%) and centrifuged at $1{,}500 \times g$ for 15 min. The resulting supernatant was discarded, and the pellet was resuspended in 0.1% Tween 80 solution and centrifuged again. The supernatant was again discarded, and the pellet was stored at −20 °C.

Concentrated samples were divided into two groups: Group A, containing 33 samples without degradation of PAC flakes, and Group B, containing 33 degraded samples. To increase the detection ability of (oo)cysts from samples, we used alkalinization to degrade flakes. Two grams of previously obtained pellet were transferred to glass jars and subjected to constant stirring on a magnetic stirrer; we used 25 μL of NaOH (10 M) per 1 g of flocculated solid (50 μL total) and stirred for 2 min. After identifying that the solutions with de-structured flakes had pHs ranging from 10 to 12, we adjusted the pH to 6–7 using HCl (5 M). The homogeneous solution was stored at −20 °C.

For DNA extraction, we used 200 μL of the sample and the commercially available NucleoSpin Tissue® kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's protocol, adding three freeze-thaw cycles for higher efficiency in (oo)cyst wall degradation. To detect *Cryptosporidium* spp. and *G. duodenalis* DNA, nested PCR in triplicate was performed with the addition of Invitrogen® BSA (bovine serum albumin) at a concentration of 500 ng per reaction to inactivate the inhibitors present in the samples.

To detect *Cryptosporidium* spp., the 18SSU rRNA gene was amplified as described by Xiao *et al.* (1999); a fragment between 826 and 864 bp was generated. To detect *G. duodenalis*, the triosephosphate isomerase (TPI) gene was amplified as described by Sulaiman *et al.* (2003). A fragment 530 bp in length was generated. Amplified products were

visualized using 1.5% agarose gel electrophoresis and ultraviolet light.

RESULTS AND DISCUSSION

All 33 samples from Group A, subjected to different concentration methodologies, were negative for *Cryptosporidium* spp.; two SSC samples were positive for *G. duodenalis*. All 33 samples from Group B were negative for *Cryptosporidium* spp. and for *G. duodenalis*.

Regarding Group A, the negative result for *Cryptosporidium* spp. does not confirm the absence of these protozoa in the Tibagi River or the WTP. As water resources are contaminated by even a low number of oocysts, the number of oocysts could be smaller than the detection limit of the test due to the dilutive potential of the water resource and the sample volume collected (Almeida *et al.* 2015). Studies point to a lower detection rate of *Cryptosporidium* compared to the detection of *Giardia* (Kitajima *et al.* 2014; Maciel & Sabogal-Paz 2016).

The presence of *G. duodenalis* was confirmed in 6.1% (2/33) of the Group A samples, showing that there was fecal excretion of cysts by mammals, which caused detectable contamination of the river. In Brazil and Paraná, *Giardia* are endemic, with a prevalence of approximately 15% in human and pet populations (Casavechia *et al.* 2016). There is an indication that contamination of water resources was due to domestic sewage and drainage of contaminated water from livestock breeding (Aksoy *et al.* 2007). Moreover, contamination of natural water resources alerts us to the risk of the use of water for irrigation and recreation (Greinert *et al.* 2004).

Regarding the methodology used, *G. duodenalis* was present in 18% (2/11) of the SSC samples of Group A. Centrifugal concentration was the simplest and most efficient method due to the smaller number of manipulation steps, resulting in less loss of cysts (Robertson *et al.* 2006). We suggest that cysts are free from PAC flakes and that they can carry over to other processes in the WTP, which was observed in the experimental trial of Maciel & Sabogal-Paz (2016) that recovered 50% of the oocysts in the supernatant of the decanted after PAC flocculation. However, the absence of *G. duodenalis* in all samples does not allow us to verify or discard this possibility.

The negative results of Group B suggested: (i) an absence of the retention of (oo)cysts in PAC flakes, contrary to studies that argue that coagulation followed by sedimentation is the most important step for retention of these protozoa in conventional treatments (Maciel & Sabogal-Paz 2016); (ii) DNA degradation, studies report the occurrence of denaturation only at an alkaline pH of 12 or below (Ageno *et al.* 1969); and (iii) the action of PCR inhibitors. We believe the PCR inhibitors is the most likely hypothesis, as studies report the inhibitory role of aluminum, a component of PAC, and other components present in the soil as humic substances (Albers *et al.* 2008; Direito *et al.* 2012; Combs *et al.* 2015). Using LAMP, as described by Karanis *et al.* (2007), the PCR inhibitors can be avoided, however the test is theoretically simple but in practice it demands advanced technical knowledge for application.

Further research on the standardization of methodologies for the processing and analysis of protozoa in samples from WTP filter residues is needed, as they can be used to improve the monitoring of these parasites in WTPs and water sources. The methods have the potential to concentrate the protozoa, which can increase the detection sensitivity and the effectiveness of prevention.

Cryptosporidium spp. was not found in settling sludge or BW samples. *G. duodenalis* was detected only in centrifuged and concentrated settling sludge samples, suggesting that this method is the simplest and most efficient method to recover (oo)cysts. Degradation of PAC flakes did not increase the detection rate for evaluated protozoa.

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