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Original Article

Surveillance of *Giardia* and *Cryptosporidium* in sewage from an urban area in Brazil

Monitoramento de *Giardia* e *Cryptosporidium* em esgoto de uma área urbana no Brasil

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Abstract

Cryptosporidium and *Giardia* are protozoan parasites that cause diarrhea in humans and animals. Molecular characterization of these pathogens in sewage may provide insight on their occurrence and prevalence in Brazil. This study aimed to investigate the presence of *Giardia* and *Cryptosporidium* in raw and treated sewage from Londrina, Paraná, Brazil. Samples were collected every two weeks during a year. Samples were concentrated, then DNA was extracted and subjected to a nested PCR targeting the *Giardia* 18S rRNA gene and the *Cryptosporidium* 18S rRNA gene. Species of *Cryptosporidium* were characterized by restriction fragment length polymorphism (RFLP). All raw sewage and 76% of the treated sewage were positive for *Giardia*; 84% of raw sewage samples and 8% of treated sewage were positive for *Cryptosporidium*. *C. muris, C. hominis, C. baileyi, C. parvum* and *C. suis* were detected in 100%, 19%, 9%, 9% and 4% of raw sewage, respectively. *C. muris* was the only species found in treated sewage. Multiple species of *Cryptosporidium* were present in 19.04% of the raw sewage. Treated sewage water can pose a threat to human health. The speciation of *Cryptosporidium* revealed the presence of non-common zoonotic species as *C. suis* and *C. muris*.

Keywords: Neglected diseases, diarrhea, environmental pollution, PCR-RFLP, raw sewage, monitoring.

Resumo

Cryptosporidium e *Giardia* são protozoários causadores de diarreia em animais e humanos. A caracterização molecular destes protozoários em esgoto pode prover dados ainda desconhecidos da ocorrência de espécies. O objetivo do presente estudo foi monitorar a ocorrência de *Giardia* e espécies de *Cryptosporidium* em esgoto bruto e tratado, em uma estação de tratamento de esgoto (ETE) de Londrina, Paraná. Amostras de esgoto bruto e tratado foram coletadas no período de um ano, com periodicidade quinzenal. A ocorrência destes protozoários foi caracterizada por meio de concentração das amostras e posterior extração de DNA seguida de *nested*-PCR para amplificação de fragmentos dos genes 18S rRNA de *Giardia* e 18S rRNA de *Cryptosporidium*. A caracterização das espécies de *Cryptosporidium* foi realizada por meio de análise por polimorfismo de comprimento do fragmento de restrição (RFLP) dos produtos obtidos. Foram coletadas no total 25 amostras de cada, esgoto bruto e esgoto tratado. Para *Giardia*, todas as amostras de esgoto bruto e 76% das de esgoto tratado foram positivas. *Cryptosporidium* esteve presente em 84% das amostras de esgoto bruto e em 8% do tratado. No esgoto tratado foi encontrado apenas *C. muris*, já nas amostras de esgoto bruto foram encontradas cinco espécies: *C. muris*, *C. hominis*, *C. baileyi*, *C. suis* e *C. parvum* em 100%, 19%, 9%, 9% e 4%, respectivamente. A presença de espécies mistas foi observada em 19,04% das amostras. A presença de *Giardia* e *Cryptosporidium* em esgoto tratado pode pôr em risco a saúde humana. A discriminação de espécies de *Cryptosporidium* revelou a presença de espécies zoonóticas incomuns como *C. suis* e *C. muris*.

Palavras-chave: Doenças negligenciadas, diarreia, contaminação ambiental, PCR-RFLP, esgoto bruto, monitoramento.

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Introduction

Cryptosporidium and *Giardia* are protozoan parasites that cause anthroponotic or zoonotic diarrheal disease which can be opportunistic or affect healthy, immunocompetent individuals. Cryptosporidiosis and giardiasis were included in a list of neglected diseases compiled by the World Health Organization (WHO), and are considered the main causes of outbreaks of waterborne protozoan disease (EFSTRATIOU et al., 2017; RYAN et al., 2014; RYAN & CACCIÒ, 2013; SAVIOLI et al., 2006).

In Brazil, these organisms have been found in a variety of samples from different sources including raw water, spring water, bottled water, treated sewage, raw sewage, sewage sludge, and vegetables (ALMEIDA et al., 2015; BONATTI & FRANCO, 2016; BRANCO et al., 2012; FRANCO & CANTUSIO, 2002; SANTOS et al., 2011; TIYO et al., 2015).

Genus *Cryptosporidium* comprises 38 species, of these *Cryptosporidium parvum* and *Cryptosporidium meleagridis* are the most frequent species associated with zoonotic transmission (FENG et al., 2018). Regarding *Giardia*, eight species have been described, out of then the species complex *Giardia duodenalis* is the one commonly described in mammals including humans, this species complex comprise eight assemblages (RYAN et al., 2019). Molecular data on the distribution of species, genotypes, and genetic assemblages of these protozoan parasites in Brazil is largely lacking in the literature, mainly due to negligence, technical difficulties, and the high cost of equipment and laboratory tests. Such data is essential to investigating the epidemiological trends, dynamics of transmission, disease patterns, and public health risks (FRANCO et al., 2012).

Molecular characterization of these pathogens in clinical samples from different hosts should be performed as it provides important taxonomic and biological data. This information can further our understanding of the epidemiology and prevalence of protozoan species or assemblages so, preventive measures and control strategies should be put into action based on these surveillance findings. Nevertheless, most of the molecular studies on *Cryptosporidium* and *Giardia* that have been carried out in Brazil analyzed animal samples (COUTO et al., 2014; DURIGAN et al., 2014; MEIRELES, 2010; NAKAMURA et al., 2009; SILVA et al., 2012, 2013).

In Brazil, cases of cryptosporidiosis due to *C. hominis* and less frequently *C. parvum* have been described in children and in immunosuppressed adults (ROLANDO et al., 2012). There are also reports, in low frequency, of *C. meleagridis*, *C. felis,* and *C. canis* infection in immunocompromised patients (ARAÚJO et al., 2008). Molecular characterization of *Giardia* in humans has mostly focused on demonstrating the occurrence of a zoonotic cycle, subassemblages of *Giardia duodenalis* frequently detected in the country are AI, AII, and BIV (COLLI et al., 2015; DURIGAN et al., 2014).

The detection of *Giardia* genetic assemblages and *Cryptosporidium* species in raw sewage samples has been used as a molecular epidemiology tool to monitor the occurrence of these protozoan parasites in the environment and to understand disease transmission dynamics (FENG et al., 2009; PLUTZER et al., 2008; ULLOA-STANOJLOVIĆ et al., 2016). Considering the environment, the characterization of these pathogens in sewage may provide insight on their occurrence and prevalence in areas without a host-based surveillance (SPANAKOS et al., 2015; XIAO et al., 2001). Nevertheless, methods able to elucidate species mixtures within a sample are necessary as the standard PCR follow sequencing only diagnostic the most abundant species (ZAHEDI et al., 2017).

The present study consisted of a one-year survey that was conducted with the aim of monitoring the occurrence of *Giardia* and *Cryptosporidium* species in raw and treated sewage samples from a wastewater treatment plant (WWTP).

Materials and Methods

Description of the study area

The study was conducted in city of Londrina (23° 18' 36" S 51° 09" 46" W) which is located in the north of the State of Paraná (PR), south Brazil. Londrina is the second largest city in the State of Paraná with a total population of 548.240, and the fourth largest city in southern Brazil. The metropolitan area has more than 1 million people, and an HDI (Human Development Index) of 0.778. Regarding basic sanitation, 89% of consumed water is treated as sewage and all sewage produced is treated. There is rainfall throughout the year. The average annual rainfall is 1429 mm (IBGE, 2015)

Characteristics of the Wastewater Treatment Plant (WWTP)

The WWTP selected is in charge of the sewage treatment in a geographic area with approximately 245.000 inhabitants. This WWTP receives an average of 3.4×10^3 m^3 of wastewater per day, performs preliminary, primary, and secondary sewage treatments consisted of railing, sand trap, primary decanter, fluidized sludge anaerobic reactors, and trickling filter, with a hydraulic retention time of 18 hours. Effluent flows into the Cambezinho stream and is categorized as class II according to the decree 357/05 of the Environment National Council (CONAMA) which categorizes waterbodies in five different classes (special, I, II, III, IV) according to water quality.

Sample collection

Samples were collected every two weeks between December 2014 and November 2015. In every sample collection day between 8:00 am and 9:00 am, 1 L of raw sewage was collected after the sand trap, and 2 L of treated sewage was collected before the runoff in Cambezinho stream, Londrina, PR, Brazil, both using a plastic jar as a sampler. A total of 25 samples of raw sewage, and 25 samples of treated sewage were collected. Samples were stored in plastic bottles which were previously sanitized and rinsed with Tween® 80 0.1%. Specimens were transported to the laboratory in isothermal boxes and processed within 24 hours after arrival.

Sample Concentration

We concentrated 500 mL of raw sewage by centrifugation (SANTOS et al., 2011). Briefly, each sample was placed in a 50 mL tube containing 5 mL of Tween® 80 1% (v/v), homogenized, and subjected to centrifugation at 1500 x g for 15 minutes in a swing rotor with no brake. The supernatant was removed with a sterile disposable pipette. The sediment was resuspended in 0.1% Tween® 80 (v/v), packed in a 50 mL tube, and further subjected to centrifugation as described above. Again, the sediment was resuspended in Tween® 80 0.1% (2-4 mL) and stored at 4°C with 50 ng of gentamicin and streptomycin and 0.02U penicillin/mL until DNA extraction.

An aliquot of 1.2 L from each sample of treated sewage was concentrated by filtration and by centrifugation as well (SANTOS et al., 2011). Briefly, samples were filtered through cellulose ester membranes (pore diameter: 1.2 µm; filter support: 47 mm diameter). Membranes were eluted in Tween® 80 0.1% (v/v) by scraping, and the eluted membranes were centrifuged at 1500 x g for 15 minutes. The sediment was resuspended in Tween® 80 0.1% (v/v) and centrifuged as mentioned above. The final sediment was resuspended in Tween® 80 0.1% (1-2mL) and stored at 4°C with 50 ng gentamicin and streptomycin and 0.02 U penicillin/mL until DNA extraction.

DNA extraction

DNA was extracted from 200 µL of sewage concentrate using the NucleoSpin Tissue® (Macherey-Nagel) commercial kit according to the manufacturer's protocol. A few modifications were introduced in the original protocol and includes 3 freeze/thaw cycles prior to the lysis step in order to promote increased rupture of cysts and oocysts (WELLS et al., 2015).

Polymerase Chain Reaction (PCR) for Giardia

A triplicate nested PCR was performed to amplify a 18S rRNA gene fragment of *Giardia* (APPELBEE et al., 2003; HOPKINS et al., 1997). The reaction consisted of 1X PCR buffer; 200 µM of each dNTP; 1.5 mM $MgCl_2$; 200 nM of each primer; 5% dimethyl sulfoxide (DMSO); 1.25 U Platinum® Taq DNA Polymerase (Invitrogen); 1.5 µL of DNA, and ultrapure water to reach a volume of 25 µL.

The product of the first round PCR was diluted in 50 µL of ultrapure water for the second round PCR. The amplification step consisted of the following cycles: 5 minutes at 95 °C followed by 35 cycles of 45 seconds at 94 °C, 45 seconds at 58 °C in the first reaction, 55 °C in the second reaction, 60 seconds at 72 °C, and 5 minutes at 72 °C. Ultrapure water was used as negative control and DNA of *Giardia duodenalis* assemblage C obtained from a dog as positive control. PCR products were submitted to electrophoresis in 1.5% agarose gel stained with SYBR Safe®, after the gels were photo documented.

Polymerase Chain Reaction (PCR) for Cryptosporidium

A triplicate nested-PCR was carried out to detect a *Cryptosporidium* 18S rRNA gene fragment ranging in length from 823 to 840 bp (XIAO et al., 2001). The reaction consisted of 1X PCR buffer; 200 µM of each dNTP; 2.5 mM of $MgCl_2$; 400 nM of each primer; 1.25 U of Platinum® Taq DNA Polymerase (Invitrogen); 200 ng/µl nonacetylated bovine serum albumin (BSA); 2.0 µL of extracted DNA, and ultrapure water to a final volume of 25 µL. The product of the first reaction was diluted with 50 µL ultrapure water before the second PCR assembly.

The amplification steps for both reactions were: 5 minutes at 95 °C; followed by 35 cycles of 45s at 94 °C; 45s at 55 °C; 60s at 72 °C and 5 minutes at 72 °C. Ultrapure water was used as negative controls, and *C. parvum* DNA obtained from a calf was used as positive control. PCR products were subjected to 1.5% agarose gel electrophoresis. Gels were stained with SYBR Safe® and photographed.

Discrimination of Cryptosporidium species by PCR-RFLP

Second round PCR products that were positive for *Cryptosporidium* were subjected to restriction fragment length polymorphism (RFLP). The enzymes *SspI*, *AseI, DdeI* and *Mboll* were used for species discrimination (FENG et al., 2007; XIAO et al., 2001). Digestions were performed separately. Each digestion consisted of 1X Buffer NEB®; 5 U of enzyme (*SspI and Mboll*) or 3 U (*AseI and DdeI*); 5 µL of the second round PCR product, and ultrapure water up to 20 µL. Samples were incubated for 1 hour at 37 °C and for 15 minutes at 65 °C. RFLP products were subjected to 2.5% agarose gel electrophoresis. Gels were stained with SYBR Safe® and photographed. Band patterns on the gels were compared with those from images available at Cryptodb (Heiges et al., 2006). In those cases, in which of co-infection was suspected, samples were compared with cleavage patterns generated by NebCutter v2.1 with reference sequences retrieved from GenBank database (accession: KT151526, GU254175, AF108864, EU331242 and DQ836341) (VINCZE et al., 2003).

Results

All raw sewage samples were positive for *Giardia*, and 84% (21/25) were positive for *Cryptosporidium*. The prevalence of these two protozoan parasites was lower in treated sewage samples; 76% (19/25) were positive for *Giardia*, and 8% (2/25) for *Cryptosporidium*.

Giardia was more frequently found than *Cryptosporidium* in the raw sewage sample replicates, 88% (22/25) of the raw sewage samples were positive for *Giardia* in all three replicates and 12% (3/25) in two replicates; 19% (4/21) of the raw sewage samples were positive for *Cryptosporidium* in all three replicates, 38% (8/21) in two replicates, 42% (9/21) in one replicate. In treated sewage *Giardia* was detected in 26% (5/19) of samples in all three replicates and 31% (6/19) in two replicates, considering *Cryptosporidium* both positive samples had only one positive replicate.

Table 1. Raw sewage samples with more than one species of *Cryptosporidium* identified by PCR-RFLP. Samples were collected bimonthly between December 2014 and November 2015 in a wastewater treatment plant (WWTP) in the city of Londrina, State of Paraná, Brazil.

Raw sewage samples	PCR-RFLP replicates	Species identification
RS6	1	*C. muris*
	2	*C. muris*, *C. hominis*
	3	*C. muris*, *C. hominis*
RS12	1	*C. muris, C. baileyi, C. suis*, *C. hominis*
	2	*C. baileyi, C. suis*, *C. hominis*
RS14	1	*C. muris*, *C. hominis*
	2	*C. baileyi, C. suis*, *C. hominis*
	3	*C. baileyi, C. suis*, *C. hominis*
RS20	1	*C. muris*
	2	*C. hominis*, *C. parvum*

Identification of the species of *Cryptosporidium* was achieved by PCR-RFLP in all positive raw sewage and treated sewage samples. Five species of *Cryptosporidium* were found in raw sewage: *C. muris*, *C. hominis*, *C. baileyi*, *C. suis,* and *C. parvum*. A high frequency of *C. muris* was detected in these samples as all samples had the pattern for this species in at least one replicate, *C. hominis* was found in 19% (4/21) of raw sewage samples. *C. suis* and *C. baileyi* were present in 9% (2/21) of these samples. We detected *C. parvum* in 4% (1/21) of raw sewage samples. Only *C. muris* was identified in treated sewage samples.

The presence of more than one species of *Cryptosporidium* in the same sample was observed in 19% (4/21) of raw sewage specimens, and included different combinations of two or three species: *C. muris*/*C. hominis; C. hominis*/*C. parvum; C. suis*/*C. baileyi*/*C. hominis,* and *C. suis*/*C. baileyi*/*C. hominis* /*C. muris*.

In all samples in which more than one species of *Cryptosporidium* was detected, there was a difference in the number of species of this protozoan parasite found depending on the replicate. The result of a replicate of one sample was inconclusive, a large number of bands in the gel made the interpretation difficult and the specimen non-diagnostic. The results of the analysis of samples in which more than one species of *Cryptosporidium* was found in PCR replicates are summarized in Table 1.

Discussion

The present survey demonstrates the occurrence of *Giardia* and *Cryptosporidium* species in raw and treated sewage samples collected in a large city from south Brazil. Previously published studies have reported the presence, in Brazil and other countries, of these two protozoan parasites in sewage (LI et al., 2012; MONTEMAYOR et al., 2005; NGUYEN et al., 2016; ROBERTSON et al., 2000; SANTOS et al., 2011; ULLOA-STANOJLOVIĆ et al., 2016). However, few studies have been able to determine the species of *Giardia* and *Cryptosporidium* that are present within the samples examined. The methods used in these studies including direct immunofluorescence (DIF), PCR and sequencing, cannot discriminate species mixtures, considering the molecular assays also the organism's viability.

The presence of *Giardia* in all raw sewage samples tested suggests that this pathogen is endemic in this geographic area, nevertheless as the PCR is designed for genus diagnostic this high occurrence can't be attributed to a specific host. In the present study, the detection of *Giardia* in treated sewage that was being released into a creek highlights that water bodies that receive treated sewage should be monitored for protozoal contamination. This is especially important when these are used in agriculture or for consume by animals and humans, thus mitigation on parasite discharge should be performed (XIAO et al., 2018). The presence of *Giardia* in humans, dogs and vegetables in Brazil has been described and through genotyping of the *Giardia* isolates a correlation between them was established, negligence in the use of water sources for irrigation favored the perpetuation of the parasite's life cycle and maintenance of the disease in the population (COLLI et al., 2015).

Method used in this study for discrimination of *Cryptosporidium* species was primarily applied to environmental samples in raw sewage samples and treated sewage receptors rivers in the United States (XIAO et al., 2001). In this study, we obtained similar results mainly about the species found in raw sewage: *C. muris, C. parvum* and *C. hominis*. PCR-RFLP has been applied in raw sewage samples in China, they have also reported the presence of *C. baileyi* and *C. suis,* and in 15.87% of the positive samples there were multiple species (FENG et al., 2009).

The ability of the PCR-RFLP to discriminate species mixtures of *Cryptosporidium* in the same sample has been demonstrated, nevertheless, it may be difficult in some samples as many species of the parasite may be present resulting in extremely mixed patterns (RUECKER et al., 2005). This was seen in only one replicate of the present study. Limiting the target sequences before testing the samples by PCR-RFLP or by PCR amplification followed by sequencing, would help, however, these molecular assays require many replicates and may result yet in extremely mixed patterns or overlapping peaks in chromatograms and also lack of amplification due to random variations of the template (RUECKER et al., 2005, 2011; SPANAKOS et al., 2015). Next generation sequence is also a promising approach to better understand the diversity of *Cryptosporidium* species within a raw sewage sample, in Australia NGS revealed a huge diversity of species in raw sewage samples, nevertheless, this tool requires a highly technified staff and in most places is an expensive tool (ZAHEDI et al., 2017).

The presence of *C. muris* in all positive samples suggests contamination of the sewage system by rodent feces. Rodents are the main reservoir of *C. muris*, and these animals are known to be present in sewage collection system (SPANAKOS et al., 2015; THOMPSON & ASH, 2016). In studies carried out in Europe and in the United States, researchers found *C. muris* in raw sewage samples and inferred that waters were contaminated by rodent feces (KHOUJA et al., 2010; SPANAKOS et al., 2015; XIAO et al., 2001). The finding of *C. muris* in treated sewage shows that this rodent pathogen occurs in wastewater treatment plants and indicates that other species of *Cryptosporidium* may be released into the environment posing a significant risk to public health. *C. muris* is described with low frequency in humans and mostly in immunosuppressed patients (CAMA et al., 2003)

Experimental studies showed that *C. muris* can cause mild diarrhea in humans resulting in persistent and asymptomatic infection (CHAPPELL et al., 2015). Thus, *C. muris* could probably circulates in the population.

C. muris was present in all positive samples, this finding may suggest that this species is more abundant underestimating other species of *Cryptosporidium* present in the same geographic area. The presence of multiple species of *Cryptosporidium* in the same sample can results in the amplification by PCR of the one that is most abundant, underestimating a possible species richness (RUECKER et al., 2011). However, in our study *C. suis, C. hominis, C. parvum* and *C. baileyi* were found in the same sample.

The occurrence of *C. suis* in sewage may occur due to nonhuman sources of fecal material discharged in domestic sewage e.g. slaughterhouse effluents, and rain runoff during rainy seasons, or *C. suis* infection occurs in the human population of the study area. Recent findings suggest that this species may be much more broadly distributed than initially thought (BODAGER et al., 2015). These findings warrant further research on the occurrence of cryptosporidiosis in healthy and immunocompromised individuals. More studies are needed in order to improve our understanding of the epidemiology by *C. muris* and *C. suis*, they are not commonly found in humans but in recent years have gained increased attention (CAMA et al., 2003; LEONI et al., 2006).

C. baileyi causes disease in birds (NAKAMURA & MEIRELES, 2015). In the present study, the presence of *C. baileyi* in sewage samples may be explained by the fact that there are a large number of synanthropic birds – especially *Zenaida auriculata* (the eared dove) - in the city of Londrina, PR, Brazil where this survey was carried out. These doves would contaminate the sewage system by runoff as they could shed oocysts in their feces.

C. parvum and *C. hominis* are the two main species that affect human beings, the former is zoonotic and the latter anthroponotic (RYAN et al., 2014). The presence of *C. hominis* in raw sewage samples indicates the occurrence of cryptosporidiosis in humans in the study area. The presence of *C. parvum* in sewage specimens may suggest the occurrence of an urban zoonotic cycle in this geographic region as this species has also been found in our area in raw water (ALMEIDA et al., 2015). Nevertheless, since *C. parvum* infects a wide range of hosts, we were unable to determine if the oocysts were of human or animal origin. The higher occurrence of *C. hominis* when compared with *C. parvum* corroborates the data on the epidemiology of cryptosporidiosis in Brazil, according to the literature, *C. hominis* infection is more frequently found than *C. parvum* (ROLANDO et al., 2012).

Conclusion

The present study documents the occurrence of *Giardia* and *Cryptosporidium* species in raw sewage and treated sewage samples from a WWTP located in the city of Londrina, State of Paraná, south Brazil. We demonstrated that *C. muris* is present in treated sewage, and *C. muris, C. hominis, C. baileyi, C. suis*, and *C. parvum* are present in raw sewage. The presence of *C. muris, C. suis* and *C. baileyi* in sewage suggests contamination with feces from rodents, pigs and birds, respectively, or human infection by *C. muris* and *C. suis*. *C. hominis* prevailed over *C. parvum* in raw sewage samples. *Giardia* high occurrence frequency confirms the endemic status of this protozoosis in the area.

Our findings highlight the problem of using water bodies that receive effluents from WWTP for human consumption and irrigation. Our study also points out that there is a need for additional research on the characterization and genotyping of species of *Cryptosporidium* that affect humans in Brazil. Therefore, the role of *C. suis* and *C. muris* in human sporadic cryptosporidiosis should be further investigated.

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