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Occurrence of *Cryptosporidium* spp. and *Giardia* spp. in a public water-treatment system, Paraná, Southern Brazil

Ocorrência de *Cryptosporidium* spp. e *Giardia* spp. em uma estação pública de tratamento de água, Paraná, Sul do Brasil

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Abstract

The purpose of this study was to investigate the occurrence of *Cryptosporidium* spp. and *Giardia* spp. in a public water-treatment system. Samples of raw and treated water were collected and concentrated using the membrane filtration technique. Direct Immunofluorescence Test was performed on the samples. DNA extraction using a commercial kit was performed and the DNA extracted was submitted to a nested-PCR reaction (n-PCR) and sequencing. In the immunofluorescence, 2/24 (8.33%) samples of raw water were positive for *Giardia* spp.. In n-PCR and sequencing, 2/24 (8.33%) samples of raw water were positive for *Giardia* spp., and 2/24 (8.33%) samples were positive for *Cryptosporidium* spp.. The sequencing showed *Cryptosporidium parvum* and *Giardia duodenalis* DNA. In raw water, there was moderate correlation among turbidity, color and *Cryptosporidium* spp. and between turbidity and *Giardia* spp.. The presence of these protozoans in the water indicates the need for monitoring for water-treatment companies.

Keywords: Sequencing DNA, nested Polymerase Chain Reaction, protozoa, water supply.

Resumo

O objetivo deste estudo foi investigar a ocorrência de *Cryptosporidium* spp. e *Giardia* spp. em um sistema público de tratamento de água. Amostras de água bruta e tratada foram coletadas e concentradas, utilizando-se a técnica de filtração em membranas. Foi realizada a técnica de Imunofluorescência Direta nas amostras. A extração de DNA foi realizada, utilizando-se um kit comercial, e o DNA extraído foi submetido a uma reação de nested-PCR (n-PCR). Na imunofluorescência, 2/24 (8,33%) amostras de água bruta foram positivas para *Giardia* spp.. Na n-PCR, 2/24 (8,33%) amostras de água bruta foram positivas para *Giardia* spp., e 2/24 (8,33%) amostras foram positivas para *Cryptosporidium* spp.. O sequenciamento demonstrou DNA de *Cryptosporidium parvum* e de *Giardia duodenalis*. Na água bruta, houve correlação moderada entre turbidez, cor e *Cryptosporidium* spp. e entre a turbidez e *Giardia* spp.. A presença desses protozoários na água indica a necessidade de monitoramento pelas empresas de tratamento de água.

Palavras-chaves: Sequenciamento de DNA, *nested Polymerase Chain Reaction*, protozoário, abastecimento de água.

Introduction

Waterborne diseases caused by protozoans have a worldwide distribution in developed and developing countries and cause epidemics and endemic diseases in humans (COTRUVO et al., 2004). Cryptosporidiosis and giardiasis are the most prevalent parasitic diseases that cause waterborne diarrhea in humans (LANE &

LLOYD, 2002). *Cryptosporidium* spp. and *Giardia* spp. produce infectious forms (oocysts or cysts, respectively) that are transmitted by the feces of the infected host and are transmitted through direct contact with contaminated food or water (CACCIÒ et al., 2005; JEX et al., 2011). Molecular methods have identified different species and subtypes of these parasites (XIAO et al., 2001), many of which are infectious to humans (CHALMERS, 2011; FAYER, 2011).

Approximately 524 outbreaks worldwide have been recorded from waterborne diseases that are attributed to protozoans. Among the etiologic agents, *Cryptosporidium* was responsible for

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285 (54.38%) of these outbreaks, followed by *Giardia*, which caused 202 (34.58%) registered cases (BALDURSSON & KARANIS, 2011).

In Brazil, the Ministry of Health Law 2914/11 outlines the procedures for the control and surveillance of water quality for human consumption and potability standards. This law determines that an annual geometric mean greater than or equal to 1,000 *Escherichia coli*/100 mL in raw water is necessary to monitor *Giardia* spp. cysts and *Cryptosporidium* spp. in different locations of the water catchment, with the goal of decreasing their occurrence (BRASIL, 2011).

There is a highest prevalence of waterborne protozoan infections in developing countries, due to their low hygiene standards, but there is no data that report the true occurrence of *Cryptosporidium* and *Giardia* in water supply sources. This lack of data leads to an underestimation of the real rate of waterborne parasite protozoan in developing countries (LIMA & STAMFORD, 2003; BALDURSSON & KARANIS, 2011).

Therefore, the purpose of this study was to investigate the occurrence of *Cryptosporidium* spp. and *Giardia* spp. in raw and treated water in a public water treatment system to perform a molecular characterization of isolates and to relate the results with physico-chemical and climate parameters.

Materials and Methods

Study area of interest

Londrina is the second largest city in Paraná State, Brazil, with a population of 537,566 residents (IBGE, 2013). Londrina is located between 23°08'47" and 23°55'46" South and between 50°52'23" and 51°19'11" West. The climate is humid subtropical, with precipitation in all seasons and an annual mean temperature of 21.6°C (LONDRINA, 2013).

There are two public water treatment systems (WTS) in Londrina; however, only one was chosen for this study. The chosen WTS performs the entire water treatment cycle (catchment, coagulation, flocculation, decantation, flotation, disinfection with chlorine, fluoridation, storage and distribution) and serves 32% of the city's population (SANEPAR, 2010).

The water is drawn from the Ribeirão Cafezal watershed. According to article 4 of Resolution No. 357 of 17 March 2005 of Conselho Nacional do Meio Ambiente (Conama), the water drawn from the Ribeirão Cafezal watershed, after conventional treatment, can be used for human consumption (BRASIL, 2005). The Ribeirão Cafezal watershed is located between 23°16'30" and 23°22'30" South and 51°11'40" and 51°23'30" West (PEREIRA & LIMBERGER, 2002).

Samples

Samples of 100 liters of treated water and 30 liters of raw water were collected every two weeks between September 2012 and September 2013. The samples were stored in plastic containers and transported to the State University of Londrina.

Water sample concentrations

The samples were analyzed using a membrane-filtration technique with cellulose acetate membranes that had a 47 mm diameter and 1.2 µm porosity (Millipore, Brazil). A vacuum pump system was used, and a filtration rate of 4 liters per minute was defined. The protocols followed those proposed by Dawson et al. (1993), Aldom & Chagla (1995) and Cantusio & Franco (2004).

Direct immunofluorescence test

We used 10 µL aliquots of each sample to perform the direct immunofluorescence test using the Merifluor *Cryptosporidium*/*Giardia* commercial kit (Meridian Bioscience, USA) according to the manufacturer's protocol, using positive and negative controls. Simultaneously, a confirmatory test was performed with inclusion of the fluorogenic vital stain DAPI (4',6'-diamidine-2-phenylindole; Sigma Chemicals Co., USA) to reveal the morphological characters (CANTUSIO & FRANCO, 2004).

To read the immunofluorescence, we used a Nikon Eclipse epifluorescent microscope with a 450-490 nm excitation filter and a 520 nm emission filter. To read the DAPI, we used a 365-400 nm excitation filter and a 395 nm emission filter (REDLINGER et al., 2002). To identify oocysts and cysts, we used an immunofluorescence defined by a bright apple-green color, the absence of pores or appendices that had a compatible size and format: 8-12 µm in size and an oval shape for *Giardia* spp. and 3-8.5 µm in diameter, a spherical shape and the presence (not necessary) of sutures in the oocyst for *Cryptosporidium* spp. (REDLINGER et al., 2002).

After enumerating the cysts and oocysts present in the samples, a formula proposed by Cantusio & Franco (2004) was used to estimate the number of cysts and oocysts per liter.

Nested-PCR for *Cryptosporidium* spp. and *Giardia* spp.

All samples were subjected to DNA extraction using the NucleoSpin Tissue commercial kit (Macherey-Nagel, Germany), according to the manufacturer's protocol.

To detect the presence of *Cryptosporidium* spp., fragments of the 18S rRNA gene were amplified using a nested-PCR reaction. The first reaction primers (Invitrogen, Brazil) were SSU-F2 (5'-TTCTAGAGCTAATACATGCG-3') and SSU-R2 (5'-CCCATTTCCTTCGAAACAGGA-3'), which amplified a 1325bp product (XIAO et al., 1999). The second reaction primers were SSU-F3 (5'-GGAAGGGTTGTATTTATTAGAT-3') and SSU-R4 (5'-AAGGAGTAAGGAACAACCTCCA-3'), generating a fragment of 819-825bp (XIAO et al., 1999). The detection threshold of the standardized technique was 10 oocysts / mL.

A nested-PCR reaction was amplified to detect *Giardia* spp. fragments of the 16S rRNA gene. The first reaction primers (Invitrogen, Brazil) were Gia2029 (5'-AAGTGTGGTGCAGACGGACTC-3') and Gia2150c (5'-CTGCTGCCGTCCTTGGATGT-3'), which amplified a product of 497bp (APPELBEE et al., 2003). The second reaction primers were RH11 (5'-CATCCGGTCGATCCTGCC-3') and RH4 (5'-AGTCGAACCCTGATTCTCCGCCAGG-3'), which

generated a fragment of 292-297 bp (HOPKINS et al., 1997). The detection threshold of the standardized technique was approximately 2 cysts / ml.

The PCR products were subjected to electrophoresis on agarose gel (Invitrogen, Brazil) 1.5% stained with SYBR® Safe (Invitrogen, Brazil) for 45' for *Cryptosporidium* spp. and 30' for *Giardia* spp.. The products were visualized using ultraviolet light and photodocumented using the program LPix Image ST (Loccus Biotechnology, USA).

The positive bands were extracted from the gel and purified using the commercial kit QIAquick Gel Extraction. Sequencing was performed using an ABI3500 sequencer Genetic Analyzer (Applied Biosystems, USA), with the support of the BigDye Terminator v3.1 Cycle Sequencing commercial kit (Applied Biosystems, USA). Both forward and reverse strands were sequenced. The nucleotide sequences obtained were compared with the standard *Cryptosporidium* and *Giardia* sequences deposited in Genbank using the BLAST System (Basic Local Alignment and Search Tool) and by manual alignment using the BioEdit program (Biological Sequence Alignment Editor). The sequences obtained were deposited in Genebank under number KP334118 and KP334117 for *Cryptosporidium* and for *Giardia* under number KP334119 and KP334120.

Physico-chemical parameters

The turbidity was calculated using a HACH turbidimeter 2100Q, the pH was measured using a pH meter DM-Digimed 2P and the color was calculated using a HACH spectrophotometer DR/890 Colorimeter.

Climate parameters

Climate data (precipitation, relative humidity and temperature) were taken from the Instituto Agrônômico do Paraná site (IAPAR, 2013).

Statistical analysis

To verify the correlation between the presence of *Cryptosporidium* spp. and *Giardia* spp. with water physico-chemical parameters and seasonality, the Pearson Correlation Coefficient (r) was calculated using the BioEstat 5.0 statistical package. The intensity correlation was defined as follows: weak ($0 < r < 0.40$), moderate ($0.40 \leq r < 0.7$) and strong ($0.70 \leq r \leq 1.0$) (FIGUEIREDO & SILVA, 2009).

Results

A total of 48 water samples, 24 raw and 24 treated, were analysed. Considering the results of the direct immunofluorescence, *Cryptosporidium* spp. was not detected in the raw or treated water samples. However, *Giardia* spp. was detected in 8.33% (2/24) of the raw water samples and was absent in the treated water samples. The *Giardia* spp. concentrations were 0.42 cysts per liter and 4.2 cysts per liter.

According to the results of nested-PCR, *Cryptosporidium* spp. was present in 8.33% (2/24) of the raw water samples. In the nested-PCR, *Giardia* spp. was found in 8.33% (2/24) of the raw water. All treated water samples were negative for both parasites. These results were confirmed by means of sequencing, which showed 100% similarity to *Cryptosporidium parvum* (GenBank: KJ469985.1 and KP004204.1) and 100% to *Giardia duodenalis* (GenBank: KJ027400.1 and AB569371.1).

For the physico-chemical parameters, the means [\pm standard deviation] of the pH, color and turbidity in the raw water were 9.55 [± 12.24], 250.54 [± 59.20] and 1198.85 [± 59.26], respectively; the means in the treated water were 6.38 [± 0.22], 4.91 [± 1.23] and 0.40 [± 0.1], respectively. The means of the temperature, relative humidity and precipitation were 21.46 [± 4.17], 70.43 [± 4.16] and 13.51 [± 6.95], respectively.

Correlations were found between *Cryptosporidium* spp. presence and both color ($r = 0.4491$, $p = 0.0277$) and turbidity ($r = 0.5422$, $p = 0.0061$) and between *Giardia* spp. presence and turbidity ($r = 0.5809$, $p = 0.0029$) (Table 1).

Discussion

This study identified the presence of *C. parvum* and *G. duodenalis* in the water supply source studied. We obtained a similar result to those of other studies that have demonstrated the occurrence of protozoans in water supply sources in different regions around the world (NISHI et al., 2009b; CANTUSIO et al., 2010; JÚLIO et al., 2012; MAHMOUDI et al., 2013).

Genetic sequencing showed the presence of *C. parvum* in positive raw water samples. *C. parvum* is considered zoonotic because it has a lower host specificity, and it is thus of considerable interest to public health (FAYER, 2004). In China, Xiao et al. (2013) found oocysts of this parasite in 86.4% of water catchment areas and Lee et al. (2014) reported its prevalence in 23.1% of Malaysian rivers. In Brazil, Araújo et al. (2011) described the occurrence of

Table 1. Pearson's Correlation Coefficient (r) between the physico-chemical and climate parameters for *Cryptosporidium* spp. and *Giardia* spp. in raw water from a public water treatment system in Londrina, Paraná, Brazil, September 2012 and September 2013.

Parameters Analyzed	<i>Cryptosporidium</i> spp.	<i>Giardia</i> spp.
pH	p= 0.7535 r= -0.0676	p= 0.7803 r= -0.0600
Color	p= 0.0277 r= 0.4491*	p= 0.0621 r= 0.3863
Turbidity	p= 0.0061 r= 0.5422*	p= 0.0029 r= 0.5809*
Temperature	p= 0.2929 r= 0.2238	p= 0.0461 r= -0.4108
Humidity	p= 0.9336 r= -0.0179	p= 0.3328 r= 0.2065
Precipitation	p= 0.6157 r= -0.1079	p= 0.6157 r= -0.1079

*significant correlation.

this parasite in 30% of the water samples collected in state of São Paulo, whereas Osaki et al. (2013) found *Cryptosporidium* spp. in 2/4 of the water catchment systems in Curitiba, Paraná State.

Different studies involving the molecular analysis and sequencing of positive samples for *Cryptosporidium* spp. have been conducted with the goal of better understanding the environmental epidemiology of this parasite. Many studies describe the occurrence of *C. meleagridis*, *C. hominis*, *C. parvum* and *C. andersoni* in water samples (ARAÚJO et al., 2011; LEE et al., 2013; RUECKER et al., 2013).

It is also possible to quantify the degree of water contamination using direct immunofluorescence. It was observed concentrations of *Giardia* spp. cysts of 0.42 cysts per liter and 4.2 cysts per liter in two samples of raw water. The infectious dose for humans is relatively low: 10 cysts can begin an infection (STEINER et al., 1997).

Genetic sequencing showed the presence of *G. duodenalis* in positive raw water samples. There are six known species of *Giardia*, but only *G. duodenalis* has a wide host range and is the only species of interest to public health (THOMPSON & MONIS, 2004), highlighting the importance of the findings of this study. *Giardia* spp. have a significant public health impact because, in addition to the prevalence and disease treatment costs, it can cause large outbreaks and affect the growth and cognitive development of children (FENG & XIAO, 2011).

Descriptions of the occurrence of *Giardia* spp. in water samples have been reported. It has been found in 92.3% of river water, 55.5% of water reservoirs and 45.2% and 26.9% of raw water from small and conventional water treatment companies, respectively, and in 19.2% of small water treatment systems and 26.8% of tap water from municipalities that performed only water chlorination (CARMENA et al., 2007).

Sequencing positive water samples for *Giardia* spp. has also been conducted to determine the species involved: *G. duodenalis* has been described in cisterns containing drinking water and in an outbreak of diarrhea in humans due to the consumption of contaminated drinking water (AHMED et al., 2010; CHEUN et al., 2013).

In recent years, interest has grown in the zoonotic transmission of *C. parvum* and *G. duodenalis*, especially in relation to their transmission caused by cattle and other livestock (COKLIN et al., 2007). This finding explains the occurrence of both protozoans in the sources examined because many dairy cattle reside along the Ribeirão Cafezal, until it arrives at the WTS analyzed.

This study found a moderate correlation between the *Cryptosporidium* spp. presence and both color and turbidity in raw water and between *Giardia* spp. presence and turbidity in raw water. Correlations between the parameters analyzed and the presence of *Cryptosporidium* spp. and *Giardia* spp. were also described by Ahmad et al. (1997), Cantusio & Franco (2004), Lopes (2009) and Nishi et al. (2009a). Low turbidity values are fundamental to obtaining better performance in the disinfection process inside water treatment systems (CERQUEIRA, 2008).

High concentrations of these two protozoa in raw water have been correlated with high concentrations or frequent detections in treated water. Thus, monitoring raw water should be an important tool for controlling and monitoring treated water quality (HELLER et al., 2004). Furthermore, *Cryptosporidium* and *Giardia* are refractory to the conventional treatments used in

water treatment plants, which makes them even more important to public health (KARANIS et al., 2007). Thus, it is critically important to determine any sources of contamination and maintain water quality (FAYER, 2004; THOMPSON & MONIS, 2004; AUSTIN et al., 2012).

The United States Environmental Protection Agency (USEPA) proposed Method 1623 to detect *Cryptosporidium* and *Giardia* in water. It is internationally recognized for its elevated efficiency. Method 1623 includes concentration phases, immunomagnetic separation and immunofluorescence microscopy (USEPA, 2005). However, at the present moment, there is not a globally preferred method for detecting *Cryptosporidium* and *Giardia* in water (FREGONESI et al., 2012).

In conclusion, this is the first report of the occurrence of *C. parvum* and *G. duodenalis* in the water supply of Londrina, Paraná State, Brazil. The circulation of *Cryptosporidium* spp. and *Giardia* spp. in the study area enhances the necessity for water treatment companies to comply with Ministry of Health Law No. 2914/11 to ensure a safe water supply for the population and avoid possible future waterborne outbreaks and negative impacts on public health.

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