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Environmental Monitoring of Opportunistic Protozoa in Rivers and Lakes: Relevance to Public Health in the Neotropics

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1. Introduction

Water is a natural resource of vital importance to living beings, but due to anthropic action several microorganisms are disseminated into aquatic environments. In developing countries, over one billion people do not have access to clean, properly treated water and approximately three billion people do not have access to adequate sanitary facilities (Kraszewski et al., 2001) This scenery is probably a consequence of the increased environmental degradation, depletion of water resources, and constant contamination of bodies of water with wastewater and industrial effluents (Pedro & Germano, 2001), causing microorganisms from soil, faeces, decomposing organic matter, and other pollutant sources to spread into water.

Goiania, the capital of the state of Goiás, located in the Midwestern Region of Brazil, has ca. 1.221.654 inhabitants and is considered a regional metropolis, among the major Brazilian cities that receive a large number of migrants (Alves & Chaveiro 2007). As a result, the city faces problems of disorderly and unsustainable urban growth with a consequent increase in superficial waste, which is a continuous source of contamination of water courses.

The current sources of public water supply for the city of Goiania, the Meia Ponte river basin and its tributary river João Leite, are constantly submitted to degradation processes due to anthropic action, such as agriculture, intensive livestock production, and urbanization. And although all the water supplies of Goiânia come from this basin (52% from the Joa˜o Leite River and 48% from the Meia Ponte River), this municipality is its largest polluter (Silva et al., 2010).

Among the microorganisms that contaminate the aquatic environment, special attention should be given to opportunistic protozoa, such as Coccidea (*Cryptosporidium parvum*, *Isospora belli*, *Sarcocystis* sp., and *Cyclospora* sp.) and Microsporidia that infect the

gastrointestinal tract, are considered emergents (Gomes et al., 2002), and also *Giardia* sp., which causes diarrhea episodes (States et al., 1997), can be spread through water.

The magnitude of enteric protozoan to public health should be emphasized because of their high prevalence, cosmopolitan distribution, and deleterious effects on the individuals' nutritional status and immune system. Although children are the most susceptible individuals to these pathogens, they also affect people from other age groups (Geldreich, 1996), mainly in subtropical and tropical areas.

According to Fayer et al. (2000) the *Cryptosporidium* is a protozoan parasite of vertebrates that causes diarrhea in humans in Different Geographical Regions of the world. Through molecular techniques, it is accepted that the *C. parvum* comprises at least two genotypes: 1 or H - only infectious for humans (anthroponotic), 2 or C - infecting cattle, men and various animals, confirming the zoonotic potential initially attributed to protozoa (Kosek et al. 2001).

Among the various water-borne pathogens (viruses, bacteria, fungi and parasites) are noted protozoa *Giardia duodenalis* (synonym *Giardia lamblia* and *Giardia intestinalis*) Thompson (2000) and *Cryptosporidium* sp., which cause gastroenteritis in humans and animals. These infectious agents are derived mainly from infected people and other warm-blooded animals, which undoubtedly pollute water (Gomes et al., 2002), highlighting some that are considered emerging, such as coccidia, *Cryptosporidium parvum, Isospora belli, Sarcocystis* sp., *Cyclospora* sp. and *Microsporidia* sp. (Garcia-Zapata et al., 2003).

For many years, *C. parvum* was considered the only emerging agent of opportunistic human infection. Recently, using molecular techniques was possible to prove that other animals and other genotypes also affect humans, such as *C. felis* (Caccio et al., 2002), *C. Muris* (Katsumata et al., 2001) or *C. meleagridis* (Pedraza-dias et al., 2000), thus showing that other species may also have an impact on public health, especially for people with immune system changes, such as patients infected with the AIDS (Acquired Immunodeficiency Syndrome), transplant recipients or patients undergoing chemotherapy, diabetics, elderly and very young children (Fayer et al., 2000). In developing countries, over one billion people do not have access to clean, properly treated water and approximately three billion people do not have access to adequate sanitary facilities (Kraszewski, 2001). This scenery is probably a consequence of the increased environmental degradation, depletion of water resources, and constant contamination of bodies of water with wastewater and industrial effluents (Pedro & Germano, 2001), causing microorganisms from soil, faeces, decomposing organic matter, and other pollutant sources to spread into water.

The magnitude of enteric protozoan to public health should be emphasized because of their high prevalence, cosmopolitan distribution, and deleterious effects on the individuals' nutritional status and immune system. Although children are the most susceptible individuals to these pathogens, they also affect people from other age groups (Geldreich, 1996), mainly in subtropical and tropical areas.

Criptosporidiosis is an important parasitic disease that can become a public health problem (Cimerman et al., 2000). The main modes of *Cryptosporidium* sp. transmission are frequently associated to contaminated water, which could be either treated or non-treated superficial water, treated water contaminated along the distribution systems, or inappropriate treated water, usually using only a simple chlorination method (Solo-gabriele & Neumeister, 1996).

Human health is likely to be affected either directly by drinking water contaminated with biological agents such as bacteria, viruses, and parasites, indirectly by consuming food or drinks prepared with contaminated water, or accidentally during recreational or professional activities.

A massive waterborne outbreak of cryptosporidiosis occurred in 1993, in Milwaukee, Wisconsin, in the United States. Approximately 403.000 people experienced illness, 4.400 of them were hospitalized, and 100 deaths were registered (Corso et al., 2003). In 1996, the United States American Environmental Protection Agency (U.S. EPA) started a program to identify, standardize, and validate new methods for the detection of *Giardia* sp. cysts and *Cryptosporidium* sp. oocysts in water environments.

From 1984 to 2000, 76 outbreaks of waterborne *Cryptosporidium* sp. have been associated with in countries like USA, England, Northern Ireland, Canada, Japan, Italy, New Zealand and Australia, affecting about 481.026 people, of these 59.2% were related to drinking water and 40.7% to the recreational use of water (Fayer et al., 2000; Fricker et al. 1998; Glaberman et al., 20; Howe et al., 2002). The most frequent causes of contamination are due to operational failures of treatment systems and water contact with sewage or faecal accident in the case of recreational waters In the U.S., factors such as deterioration in raw water quality and decrease the effectiveness of the process of coagulation and filtration of one of the local water supply companies showed an increase in turbidity of treated water and inadequate removal of *Cryptosporidium* sp. (Kramer et al., 1996).

Programs to monitor these pathogens in water have been spontaneously carried out in some countries such as the United States and the United Kingdom (Clancy et al., 1999). Since this, methods 1622 and 1623 (USEPA, 1999) have been used as reference procedures in the United States (Clancy et al., 2003; Franco, 2004).

In Brazil, the concern about water quality prompted the Health Ministry to issue one Decree - Ordinance 518 (Brasil, 2004) - establishing procedures and responsibilities regarding the control and surveillance of water quality for human consumption and pattern of potability, and other measures. Nowadays, in Brazil, routine monitoring of protozoa is not performed in bodies of water used for the abstraction of water intended for human consumption. Nonetheless, the Brazilian Health Ministry recommends the inclusion of methods for the detection of *Giardia* sp. cysts and *Cryptosporidium* sp. oocysts aiming to reach a standard in which the water supplied to the population must be free of these pathogens.

It should be emphasized that the detection of cysts and oocysts in superficial water is a crucial component to control these pathogens. However, the current methods present high variability of recovery efficiency of *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts (Hsu et al., 2001), leading to the need of aggregating other types of methodology to guarantee that water potability achieves a higher degree of reliability. Due to lack of specific techniques for detection of Microsporidia and Coccidea in water and food, the analysis has been carried out by adaptations of methods used for clinical testing (Thurston-enriquez et al., 2002).

The goal of this study was to optimize and use parasitological and molecular techniques in the analysis and seasonal monitoring of opportunistic protozoa in water from fluvial systems for human usage in the municipality of Goiânia, the capital of the state of Goiás, in

the Midwestern Region of Brazil, focusing on *Cryptosporidium* sp., *Cyclospora cayetanensis, Isopora belli* and Microsporidia.

2. Materials and methods

This is a descriptive observational study approved by the Human and Animal Research Ethics Committee at Hospital das Clínicas of Universidade Federal de Goiás.

2.1 Spatial and temporal sample delimitation

A total of 72 samples were collected on a monthly basis for one year (February 2006 to January 2007), from one point in the center of each of the following bodies of water: Meia Ponte river, João Leite river, Vaca Brava Park lake, Bosque dos Buritis lake.

Meia Ponte river

In this river two sites were selected for sampling: the first, 1 km after the emission of wastewater treated by the municipal wastewater treatment plant of Goiânia, located at 16°37'40.94"S latitude and 49°16'13.41"W longitude (MP1), and the second, located at 16°38'22.39"S latitude and 49°15'50.68"W longitude (MP2) (Figure 1).

Fig. 1. Photograph of Meia Ponte river at the time of sampling during the rainy season, showing the high volume of water and its coloring (Santos et al., 2008).

João Leite river

In this river two sites were selected for sampling: one located at 16°37'40.18"S latitude and 49°14'26.08"W longitude (JL1) (Figure 2), when this body of water reaches Goiânia, and the other located at 16°19'37.52"S latitude and 49°13'24.53"W longitude (JL2), before Goiânia. Figure 3 shows hydrographic map with the four sampling points in the rivers under study: João Leite (JL1 and JL2) and Meia Ponte (MP1 and MP2).

Fig. 2. João Leite river upstream of Goiania, after interbreeding Jurubatuba stream with the Posse stream, municipality of Goianapolis (Santos et al., 2008).

Vaca Brava Park lake

This park encompasses an area of approximately 72.7 thousand m², distributed among green areas, walking and jogging tracks, sports courts, playground, and exercise facilities. The site selected for sampling is located at 16°42'31.18"S latitude and 49°16'15.67"W longitude (VB) (Figure 4).

Bosque dos Buritis lake

Bosque dos Buritis is an urban park encompassing an area of approximately 125 m² with three artificial lakes supplied by Buriti stream. The site selected for sampling is located at 16°40'58.51"S latitude and 49°15'38.35"W longitude (BB) (Figure 5)

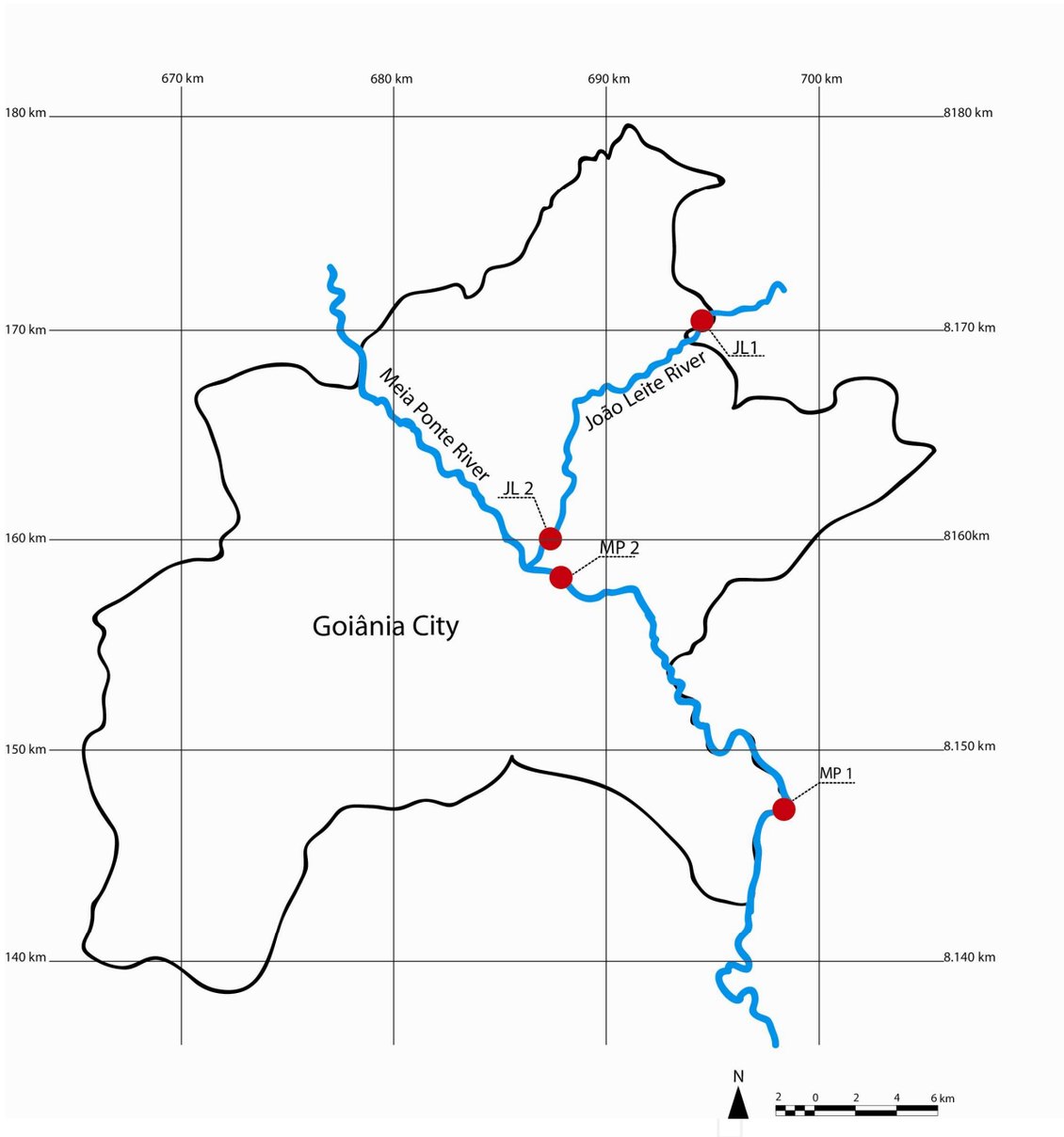

Fig. 3. Hydrographic map showing the four sampling points in the rivers under study: João Leite (JL1 and JL2) and Meia Ponte (MP1 and MP2).

Fig. 4. Photography of Vaca Brava lake, demonstrating the puopulsion system of water (Santos et al., 2008).

Fig. 5. Bosque dos Buritis lake, where we observe the dark Green water (an indicator of eutrophication) (Santos et al., 2008).

2.2 Sample concentration

Each sample was taken in a clean 10-L polyethylene container from one point in the center of the bodies of water approximately 20 cm under the surface and sent within 2 h to the Laboratório de Genética Molecular e Citogenética (Genetics and Molecular Diagnostic Laboratory) of the Universidade Federal de Goiás, and concentrated according to Silva et al. (2010).

Briefly, water samples were pre-filtered in a vacuum filter with qualitative paper filter, a process also called clarification, aiming to remove excessive amounts of organic matter, such as algae, plants, and other organisms, and immediately submitted to microfiltration using a positively nylon membrane with 0.45µm porosity with 47 mm of diameter (Hybond TM-N+, Amersham Pharmacia). The material adsorbed to the membrane was eluted by 5 ml of TE buffer (10 mM Tris-HCl, pH 8.0; 1mM EDTA) and 0.02% Tween-20, aliquoted and stored at -20°C.

2.3 Parasitological analysis

Aliquots of 10 µL of concentrated material were employed to prepare smears in two series of two slides each using the modified Ziehl-Neelsen-stain technique and the Kinyoun hot staining method, fixed in alcohol 70%, and processed for specific detection of Coccidea (*Cryptosporidium* sp., *Isospora belli*, and *Cyclospora caytanensis*).In order to detect enteral Microsporidia, the modified hot-chromotrope technique was used (Kokoskin et al., 1994). All the slides were analyzed in duplicate using a common optical microscope with a 100x oil immersion objective.

2.4 DNA extraction and amplification

The modified method of Boom et al., (1990) was used to extract the genetic material, based on cationic exchange resin processes, simultaneously with the phenol/chloroform method of Sambrook & Russel (2001).

The detection of DNA was performed using Nested-PCR, a variation of the polymerase chain reaction (PCR). The literature was searched to find primers flanking site-specific regions of these opportunistic protozoan genomes (Table 1). The Nested-PCR method was applied only to the positive and/or doubtful samples detected by parasitological methods.

Three primer pairs were used: XIAF/XIAR (*Cryptosporidium* sp. and *C. parvum*), flanking a region of approximately 1325 bp; AWA995f/AWA1206R *(Cryptosporidium* sp.*),* amplifying a region of approximately 211 bp; LAX469F/LAX869R (*C. parvum),* amplifying a chromosomal region of approximately 451 pb.

A conventional PCR was carried out using primers XIAF/XIAR and two aliquots were taken from the resulting product, one for detection of protozoan genera via Nested-PCR, using primers AWA995f/AWA1206R, (Awad-el-Kariem, 1994) and the other for the detection of *C. parvum/C. hominis* using primers LAX469F/LAX869R.

PCR using primers XIAF/XIAR and 28 µL extracted DNA was performed in a final volume of 50 µL with the following reagents: 5.0 µL buffer 10X, 2.0 mM Mg, 200 µM dNTP (dATP, dCTP, dTTP, and dGTP), 0.5 µM of each primer, and 1.25 U Taq DNA polymerase. The reaction conditions were an initial denaturation step for 4 min followed by another denaturation step of 35 cycles of 94°C for 1 min, annealing at 55°C for 45 s, extension at 72°C for 1 min, and final extension at 72°C for 7 min (Xiao, et al., 1999).

Microorganism	Primer	Sequence
Cryptosporidium sp. and *C. parvum*	XIAF	5′-TTCTAGAGCTAATACATCCG-3′
	XIAR	5′-CCCATTTCCTTGAA ACAGGA-3′
Cryptosporidium sp.	AWA995F	5′-TAGAGATTGGAGGTTGTTCCT-3′
	AWA1206R	5′-CTCCACCACTA AGAACGGCC-3′
C. parvum	LAX469F	5′-CCGAGTTTGATCCAAAAAGTTACGA-3′
C. hominis	LAX869R	5′-TAGCTCCTCATATGCCTTATTGAGTA-3′

Table 1. Primers selected to be used in confirmation/specification of protozoa detected by parasitological methods

PCR using primers AWA995f/AWA1206R and 14 µL DNA amplified by primers XIAF/XIAR was performed in a final volume of 25 µL with the following reagents: 2.5 µL buffer 10X, 1.5 mM Mg, 200 µM dNTP (dATP, dCTP, dTTP, and dGTP), 0.5 µM of each primer, and 1.25 U Taq DNA polymerase. The reaction conditions were an initial denaturation step for 7 min followed by another denaturation step of 40 cycles of 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 3 min, and final extension at 72°C for 7 min.

PCR using primers LAX469F/LAX869R Laxer, (1991) and 14 µL DNA amplified by primers XIAF/XIAR was performed in a final volume of 25 µL with the following reagents: 2.5 µL buffer 10X, 2.0 mM Mg, 200 µM dNTP (dATP, dCTP, dTTP, and dGTP), 0.5 µM of each primer, and 1.25 U Taq DNA polymerase. The reaction conditions were an initial denaturation step for 7 min followed by another denaturation step of 40 cycles of 94°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 7 min.

The PCR products were separated by electrophoresis on 8% acrylamide gels stained with silver nitrate and on 1.5% agarose gels stained with ethidium bromide. Samples presenting 211-bp and 451-bp bands were considered positive.

2.5 Direct immunofluorescence assay kit

One aliquot of each sample concentrate was tested employing the MERIFLUOR® direct immunofluorescence assay kit using homologous monoclonal antibodies for the detection of *Cryptosporidium* sp. and *Giardia* sp. Each sample was analyzed in duplicate; however, due to a shortage of reagents, this technique was applied to 50% (36/72) of the samples taken at random and the positive samples detected by parasitological methods.

2.6 Statistical analyses

The results obtained in this study were digitalized in spreadsheets using the software Microsoft Office Excel 2007. Statistical analyses were performed using the chi-squared test and the logistic regression analysis. Statistical significance level was set at $p \leq 0.05$ using the Statistical Package for the Social Sciences (SPSS) version 10.0.

3. Results

Among the 72 samples processed, 8.33% (6/72) were positive for the protozoa researched. Using the MERIFLUOR® direct immunofluorescence assay kit, we found six positive

samples: two at JL2 in September and November, one at JL1 in August, two at MP1 in July, and one at VB in September.

Using the modified Ziehl-Neelsen-stain technique, 2.7% (2/72) samples were positive for Coccidea, and the presence of *Cryptosporidium* sp. was detected in two samples and confirmed by the MERIFLUOR® direct immunofluorescence assay kit Figure 6 shows a *Cryptosporidium* sp. oocyst and Figure 7 displays a *Cryptosporidium parvum* oocyst, which is approximately 5 µm in diameter, whereas *Cryptosporidium hominis* oocyst is approximately 4 µm in diameter.

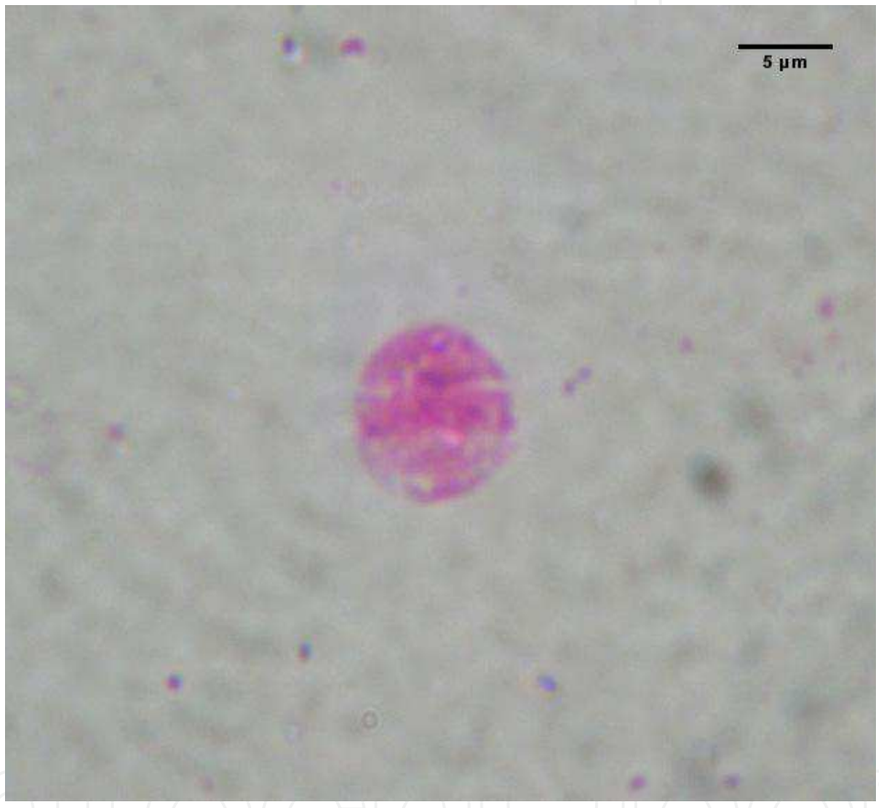

Fig. 6. *Cryptosporidium* sp. oocyst stained by the modified Ziehl-Neelsen (magnitude 100x)technique and confirmed by the MERIFLUOR® direct immunofluorescence assay kit and PCR (Santos et al., 2010).

Using primers AWA995f/AWA1206R we demonstrated that the samples belonged to the genus *Cryptosporidium* sp., and using primers LAX469F/LAX869R, we showed that just the sample collected in July was identified as *Cryptosporidium parvum*. As we detected only two positive samples for *Cryptosporidium* sp., the molecular detection was processed exclusively for them.

Using the Kinyoun hot staining method and the hot-chromotrope method for the detection of protozoa, no samples were found to be positive. Table 2 shows the results of each test carried out for the six sampling sites. Table 3 presents the frequency of protozoa detected in each sampling site.

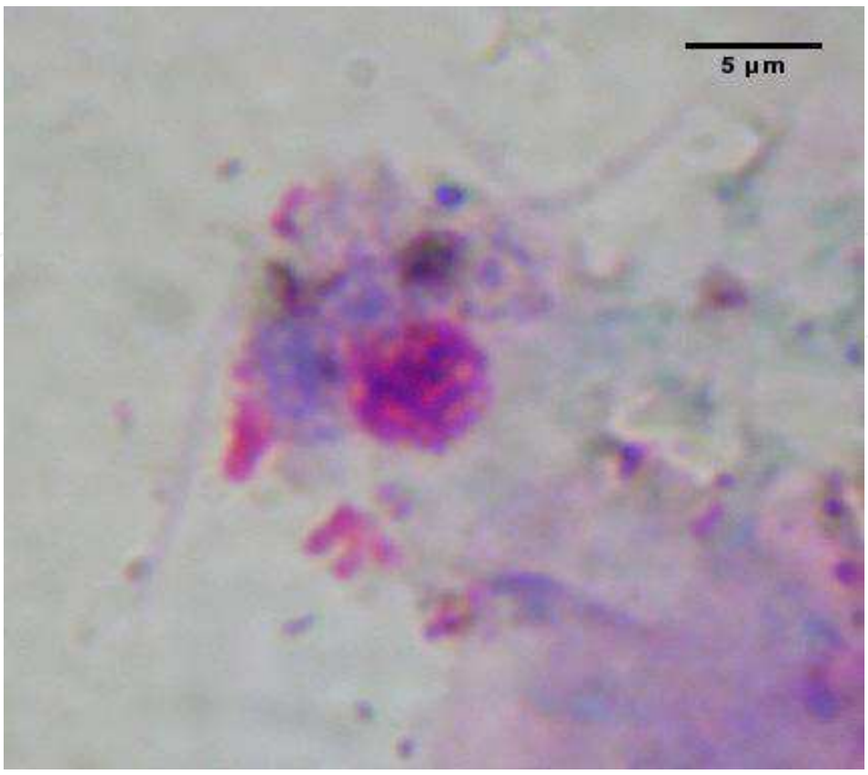

Fig. 7. *Cryptosporidium parvum* oocyst stained by the modified Ziehl-Neelsen technique (magnitude 100x) and confirmed by the MERIFLUOR® direct immunofluorescence assay kit and PCR (Santos et al., 2010).

Sampling site	Method			
	Ziehl-Neelsen	**Kinyoun**	**Hot-chromotrope**	**MERIFLUOR®**
MP1	*C. parvum**	Negative	Negative	*Giardia* sp.
MP2	Negative	Negative	Negative	Negative
JL1	Negative	Negative	Negative	*Giardia* sp.
JL2	Negative	Negative	Negative	*Giardia* sp.**
VB	*Cryptosporidium* sp.*	Negative	Negative	Negative
BB	Negative	Negative	Negative	Negative

MP1: Meia Ponte river, at 16°37'40.94"S latitude and 49°16'13.41"W longitude; MP2: Meia Ponte river at 16°38'22.39"S latitude and 49°15'50.68"W longitude; JL1: João Leite river, at 16°37'40.18"S latitude and 49°14'26.08"W longitude; JL2: João Leite river, at 16°19'37.52"S latitude and 49°13'24.53"W longitude; VB: Vaca Brava Park lake, at 16°42'31.18"S latitude and 49°16'15.67"W longitude; BB: Bosque dos Buritis lake, at 16°40'58.51"S latitude and 49°15'38.35"W longitude. *Confirmation by PCR; ** Two positive samples.

Table 2. Results according to the six sampling sites and the methods used to analyze the 12 samples in each site monitored, in a total of 72 samples (2006/2007)

Protozoa	Sampling site											
	MP1		MP2		JL1		JL2		VB		BB	
	n	%	n	%	n	%	n	%	n	%	n	%
Negative	12	100.0	10	83.4	11	91,7	10	83,3	11	91.7	12	100.0
Cryptosporidium sp.	0	0.0	0	83.4	0	0,0	0	0,0	1	8.3	0	0.0
C. parvum	0	0.0	1	8.3	0	0,0	0	0,0	0	0.0	0	0.0
Giardia lamblia	0	0.0	1	8.3	1	8,3	2	16,7	0	0.0	0	0.0
Total	12	100.0	12	100.0	12	100,0	12	100,0	12	100.0	12	100.0

MP1: Meia Ponte river, at 16°37'40.94"S latitude and 49°16'13.41"W longitude; MP2: Meia Ponte river at 16°38'22.39"S latitude and 49°15'50.68"W longitude; JL1: João Leite river, at 16°37'40.18"S latitude and 49°14'26.08"W longitude; JL2: João Leite river, at 16°19'37.52"S latitude and 49°13'24.53"W longitude; VB: Vaca Brava Park lake, at 16°42'31.18"S latitude and 49°16'15.67"W longitude; BB: Bosque dos Buritis lake, at 16°40'58.51"S latitude and 49°15'38.35"W longitude.

Table 3. General distribution of samples in the six sites according to the presence of protozoa, from February 2006 to January 2007

Average temperature in the period of protozoa occurrence was 26.8ºC, while in the period showing no register of this pathogen, it was 25.6ºC. The logistic regression analysis for temperature revealed p = 0.262 and OR = 1.227 (Table 4).
Average relative humidity in the period of protozoa occurrence was 42.3%, whereas in the period showing no register of this pathogen, it was 56.3%, a not significant value since the logistic regression analysis for relative humidity revealed p = 0.060 and OR = 0.944 (Table 4).

Protozoa	n	Mean	Standard deviation	p	OR
Temperature					
Negative	66	25.6	2.5		
Positive	6	26.8	1.5	0.262	1.227
Relative humidity					
Negative	66	56.3	16.0		
Positive	6	42.3	14.6	0.060	0.944

Table 4. Mean and standard deviation of temperature and relative humidity according to the presence of protozoa in the bodies of water sampled in Goiania during February 2006 to January 2007
(logistic regression analysis)

4. Discussion

This study revealed that the water in all sampling sites monitored during the research is not suitable for human consumption. Despite this evidence, we could observe the presence of people collecting water for human consumption, bathing, washing clothes, and even fishing. This fact is highly worrying because various waterborne diseases, not only related to opportunistic protozoa, but also to several other biological agents, can be transmitted through these contaminated bodies of water. Some sources of pollution observed in the

sampling sites were: clandestine sewage discharges, livestock and poultry farms, slaughterhouses, meat processing plants, landfills, among others.

Nonetheless, we detected low recovery efficiency of opportunistic protozoa cysts and/or oocysts, which might be related to environmental influence and physical-chemical factors, such as water pH and turbidity, among others, since the influence of physical-chemical factors on sampling was reported by other researchers (Fricker & Crabb, 1998, McCuin & Clancy, 2003). The influence of physical-chemical factors on sampling was reported by other researchers (Fricker et al., 1998; Clency et al., 2003). Adverse environmental factors have been proven to alter the morphology of cysts and oocysts (Orgerth & Stibbs, 1987) , ; thus justifying the low positivity found in the present study using parasitological methods. Other factors might have had influence as well, such as the concentration of *Cryptosporidium* sp. oocysts, based almost exclusively on particle size (Fricker, 1998). The parasitological techniques employed in our study are not specific and, consequently, concentrate a large amount of several materials that may be present in the water, such as organic and inorganic particles, bacteria, yeast, and algae, which interfere in the detection of the parasites.

However, the methods used in the present study are in accordance with those recommended for concentration and detection of microorganisms by the Standard Methods for the Examination of Water and Wastewater (Clesceri et al., 1998). They are easily applied, do not pose a great risk to the technician, and are low cost techniques, which can be employed by technicians trained to monitor water for human consumption.

Hall and Croll (1997) evaluated the performance of some rapid gravity filters in England using turbidity measurement and particle counts in filtered water as parameters for monitoring and controlling *Cryptosporidium* sp. oocysts as an indicator microorganism, a method similar to the one used in this study.

Some studies have demonstrated that *Cryptosporidium* sp. prevalence is approximately 6% in developed countries (6), around 2-6% in immunodepressed adults (Goldman & Ausiello 2004), and shows a great variation in underdeveloped countries (Casemore, 1990). In industrialized countries, the seroprevalence of oocyst antigens is between 17% and 32% (Goldman & Ausiello 2004). In Canada, a study showed that 21% of the water samples collected were contaminated with *Giardia* sp. cysts and 4.5% with *Cryptosporidium* sp. oocysts (Wallis, 1996). However, in the United States, the contamination of 65% to 97% of superficial water with *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts was reported (Kirkpatrick & Green, 1985), and it was also estimated that 80% of superficial water and 26% of treated water contains oocysts, although their infectivity has not been investigated (Goldman & Ausiello 2004). Nevertheless, we found contamination of 8.33% (6/72) of the samples in the present study, much inferior to the American data, which might be explained by the method applied. Therefore, new methodologies should be tested in order to compare the results in terms of specificity and efficiency to be employed in environmental monitoring of protozoa of public health interest.

Since our sampling points are located before the municipal wastewater treatment plant of Goiânia, the results of this study were considered within the tolerable levels, due to the low protozoan positivity according to the method used, in spite of the clandestine sewage discharges. It is worth mentioning that the water from all sources analyzed in this research is improper for usage *in natura*, because it meets neither the Brazilian standard (Brasil, 2004), which establishes that water for human consumption ought to be free from *Giardia* sp. and *Cryptospridium* sp., nor the American one (McCuin & Clancy, 2003).

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The performance of some rapid gravity filters was evaluated in England, using turbidity measurement and particle counts in filtered water as parameters for monitoring and controlling *Cryptosporidium* sp. oocysts as an indicator microorganism (Geldreich, 1996), a method similar to the one used in our study.

The *in vitro* amplification of DNA fragments of *Cryptosporidium* sp. obtained sensibility and specificity. Nevertheless, the amplification was only possible using Nested-PCR primers (AWA995f/AWA1206R and LAX469F/LAX869R). The primer LAX469F/LAX869R amplifies the regions of *C. parvum*/*C. hominis*, but *C. parvum* diagnosis was confirmed by the difference in diameter, since its oocyst is approximately 5 μm in diameter, while *C. hominis* oocyst is approximately 4 μm in diameter.

Nested-PCR presents the advantage of concentrating a smaller quantity of PCR inhibitors (Kirkpatrick & Green, 1985). In environmental samples, there are several Taq DNA polymerase inhibitors, such as fecal hemoglobin and phenolic compounds, and it might have been the case of the samples processed in the present research.

It was possible to obtain satisfactory amplification with the two methods of DNA extraction applied. Furthermore, they are quick and low-cost, although close attention should be paid to the phenol/chloroform method since it is toxic and corrosive.

As adverse environmental factors have been proven to alter the morphology of cysts and oocysts (Hsu BM, 2001), making their detection more difficult, this may justify the low positivity found in the present study using parasitological methods. Other factors might have had influence as well, such as the concentration of *Cryptosporidium* sp. oocysts, based almost exclusively on particle size (Hsu, 2001). Also, the level of protozoa may vary according to the season, and an increase in their resistant forms in rainy periods, winter and beginning of spring has already been reported (Atherholt 1998, Ong et al. 2002)

Temperature has also been considered a factor that influences protozoa and autochthonous microorganism survival in rivers (Howe, 2002). In this study, we observed just small variations of water temperature in the rivers and lakes sampled during the period of study, although within the limits that allow the survival and viability of protozoa. Using univariate logistic regression ($p = 0.066$), we demonstrated that temperature was not a statistically significant variable, whereas humidity ($p = 0.958$) was. In the region of sample collection there are two well-defined seasons, the dry (from April to September) and the rainy (from October to March) seasons, the latter characterized by torrential rain and runoff, which certainly makes the detection of parasites more difficult.

Due to the low number of protozoa found in this work, i.e. two *Cryptosporidium* sp. and four *Giardia* sp., we could not infer if the protozoan levels vary by season, but only observe the qualitative inference of their presence in the bodies of water monitored.

5. Conclusion

- The rivers and lakes of Goiânia are contaminated with opportunistic protozoa;
- Standardization and application of parasitological and molecular techniques in the analysis and seasonal monitoring of opportunistic protozoa were successfully carried out for environmental samples;

- During seasonal monitoring of opportunistic protozoa, with emphasis on Coccidia *Cryptosporidium* sp., *Cyclospora cayetanensis, Isospora belli* and Microsporidia, it was possible to detectic *Cryptosporidium parvum* and *Cryptosporidium* sp. using PCR and Nested-PCR, respectively
- The parasitological and molecular techniques applied are quick, low-cost, and can be employed in laboratories that monitor the microbiological quality of water for human consumption. Considering that the microorganisms studied herein are opportunistic, their persistent contact with humans may generate new parasites able to breach the immune barrier of normal individuals and to produce more aggressive cycles. Our results point to the need for efficient programs to prevent, treat, and monitor the presence of these parasites in rivers and lakes used for abstraction of water intended for human consumption and/or for recreational purposes all over the world. Furthermore, more efficient parasitological techniques, such as PCR, should be adopted in routine analyses in the laboratories of environmental monitoring, water for human consumption should be purified with UV radiation, and the activated sludge generated by wastewater treatment plants and intended for use in agriculture should be monitored.

6. Concluding remarks

Cryptosporidium is considered a coccidia resistant (Carey et al. 2004), because oocysts have characteristics that favor its rapid spread in the environment, such as the ability to withstand the action of commonly used disinfectants (formaldehyde, phenol, ethanol, lysol), able to cross some water filtration systems due to its small size, the ability to float, remain in the environment by a few weeks or months and tolerance in certain temperatures and salinity (Fayer et al. 2004). Given the scope of the aquatic environment coupled with the wide distribution of different species in Brazilian waters, make the control measures of Cryptosporidium limited.

Therefore, to minimize the risks inherent in the spread of cryptosporidiosis in the populations of free-living mammals, it is of fundamental importance to environmental control, through the adoption of agricultural practices to prevent pollution of rivers by the faeces of animals (Graczyk et al. 2000), as well as encouraging the adequacy of sanitation facilities, protection of water sources, education and guidance on waste discharges from vessels during nautical activities. Regarding the control measures of captive aquatic mammals, so as to minimize or eliminate the risks inherent in the spread of coccidian, several studies should be adopted.

Finally, it must be remembered that currently monitoring systems treated water are based on the frequency of fecal coliforms and Escherichia coli as indicators of pollution, and that this methodology is insufficient to predict the presence of other pathogens such as parasites. Thus, it is imperative the use of alternative methods for the diagnosis, investigation and monitoring of large amounts of water of these pathogens. For in this way can be proposed reorganization measures that contribute to reducing the incidence of opportunistic diseases emerging in water of human use, especially for children, elderly, immunocompromised and immunosuppressed patients.

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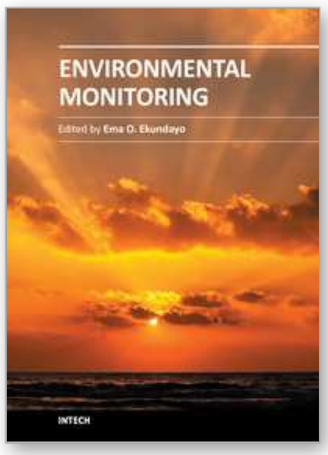

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