

Waterborne *Giardia* and *Cryptosporidium*: contamination of human drinking water by sewage and cattle feces

Veiculação hídrica de *Giardia* e *Cryptosporidium*: contaminação de água de consumo humano por esgoto sanitário e fezes de bovinos

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Abstract

Water is an important vehicle for pathogens such as *Cryptosporidium* spp. and *Giardia duodenalis*. In fact, these organisms are responsible for almost 58% and 38% of the waterborne outbreaks reported in ~60 years. Characteristics related to the environmental phase of these protozoa and the different types of hosts are important factors related to environmental contamination. In cryptosporidiosis and giardiasis, outbreaks caused by waterborne pathogens are identified as major risk factors for contamination of water resources by untreated sewage and the entrainment of cattle feces in rainwater. Further, this review covers taxonomic, biologic, and epidemiologic aspects such as prevalence, risk factors, and molecular characterization of these protozoa observed in humans and cattle in order to elucidate the role of these hosts in environmental pollution, and consequently, as a source of infection for susceptible humans, especially in rural areas. Alternative water resources such as wells and springs are used without water treatment and potability monitoring. Epidemiological data are useful, but insufficient to characterize the source of infection, thereby requiring the use of appropriate molecular methods for subtyping the protozoa detected in environmental and feces samples as well as for assessing public health risk.

Key words: Sanitation. *Giardia duodenalis*. *Cryptosporidium parvum*. Zoonosis. Rural areas. Molecular characterization.

Resumo

A água é um importante veículo de transmissão de patógenos, entre eles *Cryptosporidium* spp e *Giardia duodenalis*, respectivamente responsáveis por 58% e 38% dos surtos de veiculação hídrica registrados em cerca de 60 anos. Características relacionadas às fases ambientais destes protozoários e a grande quantidade de hospedeiros são importantes fatores relacionados à contaminação ambiental. Em surtos de criptosporidiose e giardíase por veiculação hídrica são apontados como principais fatores de risco a contaminação dos mananciais, utilizados para a dessedentação humana, por esgoto não tratado e o desague de fezes de animais devido ao carreamento por água da chuva. Além destes fatores, esta revisão aborda aspectos taxonômicos, biológicos e epidemiológicos, como prevalência, fatores de risco e caracterização molecular destes protozoários em humanos e bovinos como forma de elucidar a importância destes hospedeiros na contaminação ambiental e, consequentemente, como fonte de infecção para humanos suscetíveis, especialmente em áreas rurais. Nestas áreas são frequentemente utilizadas fontes alternativas de abastecimento de água, como poços e nascentes, sem tratamento e

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monitoramento de potabilidade. Dados epidemiológicos auxiliam, porém não são suficientes para caracterizar fontes de infecção, sendo necessário o uso de adequadas ferramentas de caracterização molecular para subtipagem de protozoários identificados em fezes e amostras ambientais e avaliação do risco de saúde pública.

Palavras-chave: Saneamento. *Giardia duodenalis*. *Cryptosporidium parvum*. Zoonoses. Rural. Caracterização molecular.

Introduction

Several parts of the world have already reported shortage of drinking water available for human and animal consumption, because of the increase in population and subsequent intervention in urban and rural environments (AMARAL et al., 2006). According to the World Health Organization's report, 1.8 billion people consume water contaminated with feces, 2.5 billion do not have access to adequate sanitation, and 1 billion defecate in open air, in 9 of every 10 rural areas (WHO, 2014).

In Brazil, despite abundance of fresh water and the knowledge that poor water quality causes public health problems, there is still a discrepancy in accessibility, because in large urban centers, supply is common, unlike small municipalities and rural areas (SILVA et al., 2012). In rural areas, ~65% of the population use alternative water supply solutions such as groundwater or deep wells and springs, without potability treatment or monitoring (BRASIL, 2015).

Sanitation infrastructure has a strong impact on public health. The discharge of sanitary sewage (treated/untreated) and drainage of animal feces into rivers favor pathogen transmission (FRANCO, 2007; SMITH et al., 2007). About 2 million tons of sanitary sewage and other effluents are released daily into water resources (UN WWAP, 2010). The biological treatment process, widely used in the world as well as in Brazil, removes most microfauna along with the organic material; however, some pathogens persist in the treated effluent (CAIN et al., 2010; TONANI et al., 2011). In addition to sanitary sewage, agricultural activities such as intensive animal breeding may interfere with the water quality

of springs (ROCHA et al., 2006). In Brazil, many rural properties do not treat agricultural residues, which are released into the environment (ORRICO JÚNIOR et al., 2012). Old and/or inadequately sealed wells, unprotected springs, destruction of riparian forest, and inadequate soil management facilitate the contamination of groundwater and surface waters in rural areas; cattle is an important source of contamination, because they may be reservoirs of zoonotic pathogens such as *Giardia duodenalis* and *Cryptosporidium parvum* (XIAO, 2010; RYAN; CACCIÒ, 2013).

Gastrointestinal diseases are directly related to the conditions of water supply, basic sanitation, and hygiene (JOVENTINO et al., 2010). Water pathogens are responsible for part of the 4 billion global cases of diarrhea and 1.6 million deaths, but improved sanitation, improvement in water quality, and family health education favor a decrease in the number of reported cases of diarrhea (FEWTRELL et al., 2005; CLASEN et al., 2007; WHO, 2009; JOVENTINO et al., 2010; SILVA et al., 2012).

Water pathogens

Although fecal bacteria were responsible for waterborne diseases until the 1980s, there has been an increase in the number of outbreaks recorded in developed countries that are caused by water protozoa such as *Giardia duodenalis*, *Cryptosporidium* spp., *Entamoeba histolytica*, *Balantidium coli*, *Sarcocystis* spp., *Toxoplasma gondii*, *Cyclospora cayetanensis*, *Isospora* spp., *Naegleria* spp., *Acanthamoeba* spp., and *Blastocystis hominis* (SMITH et al., 2006; KARANIS et

al., 2007; BALDURSSON; KARANIS, 2011; PLUTZER; KARANIS, 2016). As majority of these protozoa follow a fecal-oral route, contamination of soil and rivers by human and animal feces favors the dissemination through water (NYGÅRD et al., 2006; SMITH et al., 2007).

In the world, 936 outbreaks of waterborne protozoa were recorded between 1946 and 2016, in which *Cryptosporidium spp.* and *G. duodenalis* were responsible for about 58% and 38% of the cases, respectively (KARANIS et al., 2007; BALDURSSON; KARANIS, 2011; EFSTRATIOU et al., 2017). Developed countries such as the United States, New Zealand, and the United Kingdom have the maximum number of outbreaks recorded, probably because they have efficient surveillance and reporting agencies for waterborne outbreaks, unlike developing countries with the lowest numbers of recorded outbreaks (EFSTRATIOU et al., 2017). Among several factors contributing to the transmission of these protozoa by water, it is important to highlight the resistance of the environmental phases (cysts and oocysts) to chlorine and other chemical disinfectants conventionally used in the treatment processes. Furthermore, the characteristics related to the biological cycle of these parasites, resistance to environmental temperature, and the occurrence of species or zoonotic genotypes, which extends the range of reservoirs, thereby contributing to the dispersion of oocysts in the environment and water resources (HELLER et al., 2004; KARANIS et al., 2007). In addition to sanitary sewage, contamination of supply sources by cattle feces is a constant concern, since these animals are reservoirs of the zoonotic genotypes of *Cryptosporidium spp.* and *Giardia spp.*. Additionally, they are responsible for environmental contamination, because they eliminate a large amount of oocysts in feces, with bovine cattle increasing the possibility of outbreaks in humans (MARTINS-VIEIRA et al., 2009; WELLS et al., 2015).

Cryptosporidium: taxonomic and biological aspects

Until recently, the genus *Cryptosporidium* was classified in the Protozoa kingdom, Apicomplexa phylum, Coccidiomorpha class, Coccidia subclass, and Eucoccidiorida order because of a biological cycle and morphology similar to other coccidian protozoans (LEVINE, 1988). However, recent phylogenetic studies have shown closer proximity of *Cryptosporidium* with gregarine protozoan than with coccidia, because they have a feeding organelle and absence of apicoplast, which allowed their reclassification in the Gregarinomorpha class, Cryptogregarina subclass (KOH et al., 2013, 2014; CAVALIER-SMITH, 2014; HUANG et al., 2014; CLODE et al., 2015; ALDEYARBI; KARANIS, 2016; RYAN et al., 2016).

Cryptosporidium spp. infects the epithelial cells of the respiratory tract and gastrointestinal tract microvillitis in a wide variety of vertebrate hosts (CHALMERS; DAVIES, 2010; RYAN; XIAO, 2014). Its biological cycle is completed in only one host and the dissemination of the parasite occurs by the elimination of infectious oocysts in feces, so that the infection of susceptible hosts can occur through direct contact or by ingestion of contaminated food and water (FAYER, 2004; XIAO, 2010). Oocysts of *Cryptosporidium spp.* are approximately 2.9-8.0 μm in diameter and have the capacity of compressibility during water filtration, reaching sizes between 2 and 4 μm (FRANCO, 2007; RYAN; XIAO, 2014). They correspond to the lower exogenous stage of the Apicomplexa protozoa, which makes it difficult to study the differentiation of morphological characteristics by optical microscopy. Thus, morphology alone is not sufficient to differentiate species (FALL et al., 2003). Development of molecular tools for the characterization and phylogenetic analysis of the genus helped understand the taxonomic classification, and thus, the biology, epidemiology, and importance of different species in public and

animal health (CACCIÒ; PUTIGNANI, 2014), these tools being necessary to identify possible environmental sources of infection. Currently, 27 species of *Cryptosporidium* have been formally described and are considered valid: >60 genotypes, 17 species (*C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. cuniculus*, *C. ubiquitum*, *C. viatorum*, *C. muris*, *C. suis*, *C. fayeri*, *C. andersoni*, *C. bovis*, *C. scrofarum*, *C. tyzzeri*, and *C. erinacei*) and 3 genotype (*Cryptosporidium* horse, skunk e chipmunk I) identified in humans (RYAN et al., 2014; 2015). However, the species most frequently causing human infections include *C. hominis* (anthroponotic transmission) and *C. parvum*, a zoonotic species with high prevalence in young cattle (CACCIÒ; PUTIGNANI, 2014).

Molecular subtyping techniques such as 60-kDa glycoprotein analysis (GP60) have been widely used to study the transmission of *C. hominis* in humans and *C. parvum* in humans and ruminants. The families of *C. hominis* subtypes are identified by the Roman numeral I, followed by a letter (Ia, Ib, Id, and If), and the families of *C. parvum* are identified by the Roman numeral II, followed by a letter (IIa, IIb, IIc, and IId) (RYAN et al., 2014). Families IIa and IId of *C. parvum* have already been identified in humans and ruminants and are tagged as zoonotic, but the IIa family is the most commonly identified in cattle and humans worldwide (XIAO, 2010). The subtypes identified within family IIa differ by the number of trinucleotide sequences TCA and TCG, classified by the letters A and G, respectively. They also differ by the number of ACATCA copies immediately after trinucleotide replication, and this sequence is classified by the letter R. Thus, IIaA17G2R1 subtype represents a subtype of *C. parvum* IIa family with 17 TCA repetitions, 2 TCG repetitions, and 1 ACATCA sequence after the trinucleotides (DEL COCO et al., 2014; RYAN et al., 2014). The molecular characterization of *C. parvum* at the subtype level is important to establish the epidemiological relationship between humans and animals and to evaluate the zoonotic risk.

Epidemiological aspects of human cryptosporidiosis

In 1980s, *Cryptosporidium* spp. emerged as a cause of serious infections worldwide in people with the human immunodeficiency virus (HIV), in which, before antiretroviral therapy, was responsible for significant mortality (HUNTER; NICHOLS, 2002). Severe diarrhea is also common in children under 5 years of age and self-limiting in immunocompetent patients (SHIKANI; WEISS, 2014; RYAN; HIJJAWI, 2015).

Human cryptosporidiosis is distributed worldwide, but there is no true estimate of infections per year, because the disease is not notifiable in most countries, there is a low demand for medical care for patients with diarrhea, no adequate techniques are used to diagnose parasites in feces, and asymptomatic infections are not investigated (SAVIOLI et al., 2006). Frequency tends to vary from 2.6% to 31.5% in developing countries and from 0.1% to 14.1% in developed countries, probably because of improved sanitation and drinking water supply (FAYER, 2004). In developing countries, cryptosporidiosis is most often described in children, who are naturally susceptible to parasitic infections (ORTEGA-PIERRES et al., 2009). Studies conducted in Asia and Africa have shown that *Cryptosporidium* spp., together with *Rotavirus*, *Shigella* spp., and *Escherichia coli* is responsible for severe diarrhea and is the second leading cause of death due to diarrhea in children up to 5 years (KOTLOFF et al., 2013; STRIEPEN, 2013). In Brazil, the few studies that report infection with *Cryptosporidium* spp. in humans are restricted to children and the immunosuppressed individuals. Timely studies conducted in urban and rural areas identified prevalence rates ranging from 0.03% to 12.9%, with the highest rates being in children attending daycare centers (GONÇALVES et al., 2006; ANARUMA FILHO et al., 2007; BRANCO et al., 2011; LANDER et al., 2012; ROLANDO et al., 2012). However, in children with diarrhea, the prevalence tends to increase, with rates ranging from 18.7% to 32.4% (PEREIRA et al., 2002;

NASCIMENTO et al., 2009). Most of the Brazilian studies used optical microscopy combined with differential color methods as a screening method for *Cryptosporidium* spp., which has low sensitivity compared to molecular techniques (ELSAFI et al., 2013).

Higher frequency of *C. hominis* transmission is observed in urban environments because of the close contact between humans, and in some countries, *C. parvum* is more frequently detected in rural areas because of the differences in sources of infection, since young cattle are the main hosts of this species (XIAO, 2010; RYAN et al., 2014). In countries where *C. parvum* is frequently identified in humans, subtype IIa family causing anthroponotic transmission is the chief agent; this subtype with zoonotic potential has been identified to be the most common causative agent in rural areas (XIAO et al., 2004). The available data on the distribution of *Cryptosporidium* species in humans in Brazil demonstrate that, in the studied population, *C. hominis* is more frequent than *C. parvum*. In studies that discuss the molecular characterization of feces samples testing positive for *Cryptosporidium* spp., *C. hominis* was detected at a frequency of 40-90% in HIV-positive patients and 57.1-73.6% in children. In contrast, *C. parvum* frequency ranged from 10% to 20% in HIV-positive patients and from 10.5% to 42.9% in children (GONÇALVES et al., 2006; BUSHEN et al., 2007; ARAÚJO et al., 2008; LUCCA et al., 2009; ROLANDO et al., 2012).

Epidemiological aspects of bovine cryptosporidiosis

Bovine cryptosporidiosis can occur in all age groups, in both beef and dairy cattle, in which the prevalence rate varies considerably across studies, with a tendency to decrease with increasing animal age (SANTÍN et al., 2008; BUDU-AMOAKO et al., 2012). The *Cryptosporidium* species identified in cattle include *C. parvum*, *C. bovis*, *C. ryanae*, and *C. andersoni* (XIAO, 2010).

Studies on dairy farms have shown that *C. parvum* was more frequent in calves up to 2 months of age; *C. bovis* and *C. ryanae*, in older calves and steers; and *C. andersoni*, in steers and adults (SANTÍN et al., 2004; FAYER et al., 2007; SANTÍN et al., 2008). Despite the low prevalence of *C. parvum* in steers and adults, some studies have identified this species in this age group (FAYER et al., 2007; BUDU-AMOAKO et al., 2012; WELLS et al., 2015). In Brazil, *C. parvum* was also more frequently identified in calves up to 2 months of age (THOMAZ et al., 2007; MEIRELES et al., 2011; COUTO et al., 2014). As cattle can act as a source of infection for humans because they are important reservoirs of *C. parvum*, it is essential to gather information about the prevalence and molecular characteristics of the species and subtypes in circulation, in order to evaluate the extent of environmental contamination as well as the risks of zoonotic transmission through water.

Cryptosporidium spp. have been documented as an important cause of neonatal enteritis in cattle farms (SANTÍN, 2013; SILVERLAS; BLANCO-PENEDO, 2013). Newborn calves with diarrhea caused by this protozoan can eliminate large amounts of oocysts in feces; in the first 2 weeks of life, an infected calf can eliminate $>10^{10}$ oocysts/day, contributing to the infection of other animals as well as to environmental contamination, since oocysts can remain viable for a long time in the environment (ROBERTSON et al., 2014; WELLS et al., 2015, 2016). In addition to age, factors related to management contribute to the occurrence of infection and/or clinical signs such as collective heifers, high population density, non-stratification of animals by age, low hygiene levels of the facilities and buckets used for breastfeeding and proximity from the heifers to the barns (FEITOSA et al., 2004; ALMEIDA et al., 2008; ROBERTSON et al., 2014; DELAFOSSE et al., 2015).

Cattle has been identified as a risk factor for cryptosporidiosis in humans, and the risk increases

at sites with animals suffering from neonatal diarrhea. (HUNTER et al., 2004; SMITH et al., 2004; ROBERTSON et al., 2014). Infections with *C. parvum* occur more frequently in rural areas in the US and Europe, where intensive breeding facilitates the persistence of transmission of *C. parvum* subtype IIa (XIAO; FAYER, 2008; XIAO, 2010). In a study performed in Sweden, *C. parvum* subtype IIa had a significant association with the aqueous feces of calves, compared to subtype IIId, in addition to a higher oocyst count in the hosts (SILVERLÄS et al., 2013). In Brazil, the few studies on subtyping of bovines have identified zoonotic subtypes (MEIRELES et al., 2011; COUTO et al., 2014; HECKLER et al., 2015; TOLEDO et al., 2017). Identification of the same *C. parvum* subtype in humans and cattle may indicate a zoonotic infection, but zoonotic subtypes may propagate among the human population in the same way as anthroponotic diseases (ROBERTSON et al., 2014). Thus, identification of the species and subtypes of *Cryptosporidium* spp. isolated from animals, humans, and water from rural areas should be performed along with the collection of epidemiological data such as “feces destination” from humans and animals and other data associated with the parasite.

Giardia: taxonomic and biological aspects

Giardia is a protozoan that multiplies asexually on the surface of small intestine microvilli of vertebrate hosts (THOMPSON, 2004; RYAN; CACCIÒ, 2013). Cysts are infectious as soon as they are released into feces, and they can survive in the environment for weeks to months and can contaminate water and food (FENG; XIAO, 2011).

Currently, the genus *Giardia* is classified in the kingdom Excavata, phylum Metamonada, class Treponadea, order Diplomonadida, subfamily Giardiinae, and family Giardiidae (ADL et al., 2012). Six species are classified according to trophozoite and/or cyst morphology, but only

G. duodenalis is identified to have the ability to parasitize humans and other mammals, while *G. agilis* has been identified in amphibians, *G. ardeae* and *G. psittaci* in birds, and *G. microti* and *G. muris* in rodents (FENG; XIAO, 2011; RYAN; CACCIÒ, 2013). *G. duodenalis* is divided into 8 genetic groups or *assemblages* (A to H) according to protein characteristics and DNA polymorphisms (CACCIÒ et al., 2005; XIAO; RYAN, 2008; RYAN; CACCIÒ, 2013). Only *assemblages* A and B share human and animal hosts, and therefore, they are considered to possess zoonotic potential; *assemblages* C to H are considered specific hosts, with C and D being common in canids and ungulate animals, F in cats, G in rats and H in marine mammals (CACCIÒ; SPRONG, 2010).

Small genetic alterations have been identified in isolates of the same *assemblage*, being denominated as *sub-assemblages*, and in a same *sub-assemblage*, denominated as genotypes. Cacciò et al. (2008) proposed that *sub-assemblages* be denominated by the letter of the *assemblage*, followed by a Roman numeral (AI, AII), and that the genotypes be denominated by the identification of the *sub-assemblage* followed by an Arabic numeral (AI-1, AI-2). *Sub-assemblage* distribution studies in hosts have shown that human isolates often belong to the *sub-assemblages* AI and AII, whereas isolates from other mammals belong to the *sub-assemblages* AI, AIII, and AIV (THOMPSON, 2004; RYAN; CACCIÒ, 2013). *Sub-assemblages* AI and AII are identified in both animals and humans, with AI preferentially in animals and AII in humans. *Sub-assemblages* BIII and BIV are more frequent in humans, while BI and BII are more frequent in animals (SPRONG et al., 2009; FENG; XIAO, 2011; RYAN; CACCIÒ, 2013).

Epidemiology of human giardiasis

Infection with *G. duodenalis* is more frequent in developing countries; the asymptomatic form is the most common form, whose carriers are important

sources of infection for susceptible individuals. Studies with asymptomatic children in developed countries revealed a prevalence rate of <10%, while those in developing countries showed a rate ranging from 8% to 30% (YASON; RIVERA, 2007; FENG; XIAO, 2011). The increase in the prevalence of infection and the frequency of outbreaks in daycare centers led to the inclusion of giardiasis in the group of neglected diseases declared by the World Health Organization (SAVIOLE et al., 2006). Many studies conducted in Brazil have reported the occurrence of *G. duodenalis* infections in children, at a prevalence rate ranging from 12.4% to 73.6% (MASCARINI; DONALÍSIO, 2006; RUIZ LOPES et al., 2006; ARRUDA et al., 2008; BISCEGLI et al., 2009; SILVA, 2009; BELLOTO et al., 2011; SANTANA et al., 2014; DAVID et al., 2015). This large variation in prevalence rates might be related to the study site, age of the study population, and local sanitation conditions, with the most frequent infection being in children aged 1 to 5 years, in population agglomerations that favor interpersonal contact such as daycare centers and schools, and places lacking a basic sanitation system (SANTANA et al., 2014). A study on enteroparasitoses involving schoolchildren from Jataizinho, a municipality near Londrina, Paraná, identified the main factor associated with enteroparasitoses as bathing in rivers, lakes, or streams (RUIZ LOPES et al., 2006). In this context, considering that enteroparasites, including *G. duodenalis*, have the same route of environmental contamination and infection, the results of this study point to the importance of water as a vehicle of this parasite.

Human beings are parasitized by the *assemblages* A and B of *G. duodenalis*, with a higher global prevalence of *assemblage* B and a higher prevalence of mixed infections in developing countries than in developed countries (RYAN; CACCIÒ, 2013). *Sub-assemblage* AI has a high zoonotic potential, because it is often identified in humans, companion animals, and production animals (SPRONG et al., 2009; RYAN; CACCIÒ, 2013). The molecular

epidemiology for *G. duodenalis* in humans is poorly explored in Brazil, and there is a lack of concrete data about *assemblages* and *sub-assemblages* (COLLI et al., 2015; DAVID et al., 2015). In São Paulo state, Brazil, David et al. (2015) studied the molecular epidemiology of *Giardia* spp. in a low-income asymptomatic population and identified *G. duodenalis* in 15% humans and 12.2% dogs. When these isolates were characterized, they identified *assemblages* A and B in humans, and A, C, and D in dogs, concluding that because of the high prevalence of *G. duodenalis* and the associated genetic variability, this population was exposed to multiple sources of infection and transmission, including direct contact, contaminated water, and food intake, suggesting the importance of environmental sources and the need for genetic characterization to identify the sources of infection.

Epidemiology of bovine giardiasis

Studies in different countries have identified *G. duodenalis* in the feces of beef and dairy cattle with a prevalence rate ranging from 5.1% to 100% of animals across different countries (SANTÍN et al., 2009; OTERO-NEGRETE et al., 2011; TIRANTI et al., 2011; BUDU-AMOAKO et al., 2012; HERNÁNDEZ-GALLO; CORTÉZ-VECINO, 2012; OUCHENE et al., 2014), and 7.5-25.56% in Brazil (MARQUES et al., 2005; SILVA JÚNIOR et al., 2011; PAZ E SILVA et al., 2012). *G. duodenalis* has been found to be highly prevalent in young calves, especially those aged between 2 and 3 months of age, when stress and lower immunity are observed because of the weaning process (TROUT et al., 2005; MADDIX-HYTTEL et al., 2006; SILVA JÚNIOR et al., 2011). Young animals are considered the main source of infection for susceptible hosts, as they can release about 10^6 cysts/g of feces (GEURDEN et al., 2010). Although cyst elimination is lower at 6 months of age, cows should be considered a potential source of infection because of increased elimination of

cysts during the peripartum period (GEURDEN et al., 2012). Collective shelters and pickets, facilities with precarious hygiene and cleaning conditions and nearby heifers, and sites in the lower planes than the corral region are considered risk factors, because of the higher levels of environmental contamination in these conditions (HAMNES et al., 2006; SILVA JÚNIOR et al., 2011). Although some studies associate infection with diarrhea, it is known that diarrhea and the release of cysts in parasitized animals are intermittent, which makes it difficult to establish this association (MCALLISTER et al., 2005; OUCHENE et al., 2014).

The *assemblage* E is dominant in ruminants, and was predominant in cattle in studies conducted in Germany, the UK, Italy, the US, Canada, Australia, and Brazil (SANTÍN et al., 2009; BUDU-AMOAKO et al., 2012; GEURDEN et al., 2012; PAZ E SILVA et al., 2012; ASHER et al., 2016). Despite the fact that *assemblage* E represents high host specificity, a study conducted in Australia identified it in the feces of the residents of urban and rural areas, who presented with diarrhea, suggesting a possible zoonotic transmission of this *assemblage* (ZAHEDI et al., 2017).

Zoonotic *assemblage* A has been increasingly identified in cattle, either alone or in mixed infections (RYAN; CACCIÒ, 2013). In a study conducted across 4 European countries, Geurden et al. (2012) identified *assemblage* A in 43% and mixed A and E infection in 32% of the 942 calves testing positive for fecal *G. duodenalis*. Although cattle are more frequently parasitized by the *sub-assemblage* AI, unlike humans who are more frequently parasitized by the *sub-assemblage* AII, some studies have identified AI-1 genotype in the calves and workers of the same rural property, suggesting zoonotic transmission (XIAO; FAYER, 2008; KHAN et al., 2011). Although not common in cattle, *assemblage* B has been identified in studies conducted in New Zealand, China, and Mexico (WINKWORTH et al., 2008; LIU et al., 2012; OTERO-NEGRETE et al., 2011). In Mexico, cattle and sheep were identified

to have a mixed infection caused by 2 zoonotic *assemblages*, namely, AI and BIII (TROUT et al., 2004; GEURDEN et al., 2010; ABEYWARDENA et al., 2013).

Cryptosporidium and *Giardia*: waterborne organisms and their association with sanitary sewage and fecal matter

Cryptosporidium spp. and *Giardia* spp. are involved in waterborne outbreaks, because they are eliminated in large quantities by their hosts and are resistant to the environmental conditions and processes commonly used in water treatment (CALDERON; CRAUN, 2006; KARANIS et al., 2007). One of the primary risk factors in these outbreaks is the contamination of water sources by untreated sewage. The treated sewage might contaminate the fountains too as these protozoa are resistant to the commonly used treatment processes. In fact, an organic matter removal rate as high as 99% does not guarantee complete elimination of these protozoa (NYGÅRD et al., 2006). Contamination of water resources by animal feces mixed with rainwater has also been reported (APPELBEE et al., 2003; SMITH et al., 2007). The importance of this form of contamination varies according to the characteristics of the hydrographic basin and depends on the type of exploration activities located in the catchment area (JIANG et al., 2005). Contamination of wells and springs by the dispersion of these protozoa and contamination of public or domestic reservoirs represent danger to the population, as there are no barriers between the reservoirs and the consumer, and generally, a reservoir serves a large portion of the population (GAUT et al., 2008; PULESTON et al., 2014).

A major outbreak of human cryptosporidiosis occurred in 1993 in Milwaukee, Wisconsin (US), attracting worldwide interest to the disease, mainly because it was transmitted by water, which demonstrated the parasite's ability to resist existing water treatment methods (MACKENZIE et al.,

1995; FAYER, 2004). The outbreak affected 403.000 people who developed symptoms of gastroenteritis within 2 months of ingesting water from the supply network (CORSO et al., 2003). Despite initial suspicions around water contamination by oocysts from cattle feces from dairy farms, molecular techniques showed that the species involved in the Milwaukee outbreak was *C. hominis* from sanitary sewage (ZHOU et al., 2003).

Following this, most outbreaks recorded until 2016 were related to deficiencies in the water treatment system. (KARANIS et al., 2007; BALDURSSON; KARANIS, 2011; EFSTRATIOU et al., 2017). Although *C. hominis* is responsible for a greater number of outbreaks even in countries where *C. parvum* is prevalent, zoonotic transmission may occur if there is an increase in the number of infected reservoirs as well as in environmental contamination (GALVÁN et al., 2014). In the UK, the increase in cryptosporidiosis outbreaks has led to changes in the legislation regarding routine evaluation of the presence of this protozoan in water, according to the parameters that affect the survival of this protozoan. The presence of cattle near the basin poses a great risk, which further doubles if calves and lambs are involved (WELLS et al., 2015). As cattle is an important reservoir of this protozoan, the discharge of fecal material present in the pastures into water bodies can generate a higher concentration of oocysts than the discharge of domestic sewage, especially during months with higher rainfall (MARTINS-VIEIRA et al., 2009). In a recent study by Wells et al. (2015), in Scotland, it was possible to compare the high prevalence of *C. parvum* in cattle and deer that inhabit the region of the water resource with the history of outbreaks occurring after the ingestion of water from it, thereby suggesting cyclical and seasonal transmission.

The first recorded outbreak of waterborne giardiasis occurred between late 1965 and early 1966 in Aspen (Colorado, US), where 2 wells serving the same side of the city were contaminated by sewage, affecting about 120 people (MOORE et al., 1969).

However, the largest outbreak of giardiasis due to contaminated water intake occurred in Norway in 2004, reaching about 1500 people (ROBERTSON et al., 2006). Between 1965 and 2016, approximately 38% outbreaks involving waterborne protozoa that have been registered worldwide were caused by *Giardia* spp.; most of these can be attributed to deficiencies in the filtration process (THOMPSON, 2004; KARANIS et al., 2007; BALDURSSON; KARANIS, 2011; EFSTRATIOU et al., 2017). Cattle has already been considered as a source of water contamination; however, there is little evidence that these animals are the main source of waterborne outbreaks, and that contamination with sanitary effluent from domestic sources is the most likely source (THOMPSON, 2004; SMITH et al., 2006; THOMPSON, 2008). Livestock and rainfall in poorly filtered catchment areas favor the occurrence of outbreaks, especially in areas where environmental concentrations of these protozoans are higher (RISEBRO et al., 2007; PLUTZER et al., 2010a). Heitman et al. (2002) investigated the significance of different sources of environmental contamination over 2 years and found that although the prevalence of *Giardia* spp. cysts was higher in sewage effluents, the concentration of cysts was much higher in cattle feces. The importance of each source should be interpreted with the help of genotype identification, but the importance of these animals should be considered during the elimination of the large number of cysts in the environment, especially because they act as reservoirs of zoonotic species (THOMPSON, 2004; XIAO; FAYER, 2008; FENG; XIAO, 2011).

Although there are no documented data to prove the occurrence of cryptosporidiosis and giardiasis outbreaks and waterborne transmission in Brazil, the presence of these parasites in treated and untreated water made available for human consumption was reported by studies conducted in different regions (HACHICH et al., 2004; HELLER et al., 2004; DIAS et al., 2008; NISHI et al., 2009a, 2009b; MACHADO et al., 2009; RAZZOLINI et al.,

2010; TOLEDO et al., 2017). As the main source of contamination of public water supply include the discharge of sanitary sewage and agricultural waste, the occurrence and concentration of these parasites in water bodies in developing countries as well as in rural areas are expected to be greater (HELLER et al., 2004). Water sources close to areas of livestock exploitation are subject to greater contamination by the wastes from these properties. At one of the water supply sources of Viçosa (MG) population, located in an area of intense agricultural activity, it was verified that at some water collection sites, although the presence of *Giardia* spp. was related to the effluent discharged from the sewage treatment station, the presence of *Cryptosporidium* spp. oocysts was related to the animal breeding station nearby (DIAS et al., 2008). In this context, rural residents are expected to be at a higher risk for waterborne infections, since many properties obtain water from private sources that do not undergo treatment and potability monitoring. Nishi et al. (2009b) identified the presence of these protozoa in water samples collected from the river, springs, and artesian wells in the indigenous lands near the Ivaí River, in the central region of Paraná, Brazil, and attributed their presence to human and animal fecal matter contamination. Toledo et al. (2017) identified cysts of *Giardia* spp. and *C. parvum* oocysts in the spring water used for human consumption in dairy farms in the western central region of Paraná, where animals were carriers of these parasites. In this study, the subtype IIaA17G2R1 of *C. parvum* was identified in calf feces as well as at the property; this subtype has been associated with outbreaks of human cryptosporidiosis in previous studies (CDC, 2011).

The occurrence of protozoan outbreaks on consumption of treated water and the presence of *Cryptosporidium* spp. and *Giardia* spp. in the water sampled from public supply led to the questioning of the quality of the treatment methods used in Brazil for parasite removal, which further led to the revision of the law 518/2004. It was replaced by

the law 2.914/2011, which provides quality control and allows monitoring of water quality and its drinking standard. This establishes the standard for evaluating the presence of pathogenic protozoa such as *Cryptosporidium* spp. and *Giardia* spp. when the annual geometric average of *Escherichia coli* found in superficial rough water is ≥ 1000 cells/100 mL. In addition, when the identified concentration is ≥ 3.0 oocysts/L at the uptake, it is recommended that the effluent from the fast filtration plant has a turbidity value of ≤ 0.3 μ T in 95% of the samples, or that a disinfection process with proven efficiency in the removal of *Cryptosporidium* spp. oocysts be used (BRASIL, 2011). Thus, the residents of dairy farms or rural properties whose water for human consumption does not come from the public supply system, and therefore, does not undergo the monitoring determined by law 2914/2011, are at risk to infection by these parasites.

Molecular identification for characterization of infection sources

Many studies based on epidemiological data discuss the possible source of environmental contamination and do not undertake molecular characterization of the parasites identified in the water samples. Molecular characterization of the species and genotypes of *Cryptosporidium* and *Giardia* spp. found in environmental samples is necessary to track the origin of contamination, so that it is possible to establish the sources and routes of transmission and to determine the importance of this form of contamination in public health, which is reinforced with the information available about the species identified in the animals inhabiting the region (ROBINSON et al., 2011; RUECKER et al., 2013).

Amplification of fragments of the small RNA subunit RNA ribosomal (18S rRNA), followed by genetic sequencing or PCR-RFLP, of an 830-bp fragment of the gene and enzyme genotyping with the restriction enzymes *sspI* and *vspI* are widely

used tools for identification of *Cryptosporidium* spp. in humans, animals, and water samples (XIAO; FAYER, 2008; RYAN et al., 2014). The use of high-resolution molecular tools for the study of intra-species variation in *C. hominis* and *C. parvum* has become common in order to establish transmission between humans and between humans and ruminants (ROBINSON; CHALMERS, 2012). Analysis of the GP60 gene sequence has been used to identify families of *C. hominis* and *C. parvum* subtypes (RYAN et al., 2014); however, some studies have performed multilocus sequencing analysis of *C. parvum* by region-based analysis of microsatellites with the objective of improving the resolution, since the analysis of a “locus” may miss possible recombination occurring during the sexual phase of the parasite cycle (RYAN et al., 2014; ROBINSON; CHALMERS, 2012). A study performed in Scotland performed subtyping of the *C. parvum* isolates identified in the feces samples of cattle, sheep, and wild animals, and water samples from the public supply by using conventional analysis of GP60 and multilocus subtyping using 6 markers (MM5, MM18, MM19, TP14, MS1, and MS9). The same subtype was identified in all samples subjected to the 2 methods, but the authors concluded that the analysis was successfully performed in a few samples, probably because it was standardized for calf samples, which eliminate large numbers of oocysts, but not in samples from adult or other animals that contain smaller amount of oocysts (WELLS et al., 2015). Adult bovine animals excrete large quantities of fibrous fecal material with low oocyst count, so simple methods such as concentrating the sample in hypersaturated salt solution will not allow complete isolation, thus making the DNA extraction process difficult (WELLS et al., 2016).

In case of *Giardia* spp., molecular tools allow identification at the level of species, *assemblages*, *sub-assemblages*, and genotypes (CACCIÒ; RYAN, 2008). Extensive identification of *G. duodenalis* and its *assemblages* has been extensively performed

through the genes 18S rRNA, triose phosphate isomerase (*tpi*), glutamate dehydrogenase (*gdh*), and β -giardin. Among these, the 18S rRNA gene corresponds to a highly conserved region that features high sensitivity in identifying *Giardia* spp., but is of little use in *assemblages* studies, because they exhibit polymorphisms in very small fragments (RYAN; CACCIÒ, 2013). Many genotyping studies have used only 1 or 2 markers, which has limited the discriminatory power of *assemblages*; thus, the multilocus genotyping method offers more information with respect to parasite genotyping (LEBBAD et al., 2011). Durigan et al. (2014) used multiple markers to evaluate the genetic diversity of *G. duodenalis* in clinical samples from humans and animals and environmental samples from the metropolitan region of Campinas, SP, Brazil, and identified higher prevalence of mixed infections in animal samples, the prevalence being 100% in genotyped samples with at least 2 markers.

The techniques currently available for the molecular characterization of these protozoa were primarily developed for the analysis of fecal or isolated samples, and so caution should be exercised when they are being used for environmental samples. Low amounts of oocysts are often identified in environmental samples compared to fecal samples, involving only one round of PCR with reduced capacity to identify protozoa and species diversity as well as the genotypes present in only one sample (NICHOLS et al., 2010; RUECKER et al., 2013; DURIGAN et al., 2014). A way to improve performance in identifying these protozoa is to perform nested PCR repetitions on the same sample, followed by DNA sequencing (RUECKER et al., 2011). Ruecker et al. (2013) analyzed 5 repetitions of nested PCR from the material extracted from the same water sample and observed improvement in the identification of the species and genotypes of *Cryptosporidium* spp. in a single sample, which favored source tracking and risk evaluation. In this same study, the most frequent species was *C. andersoni*, widely identified in cattle, demonstrating

the importance of these animals in environmental contamination.

In addition to the smaller number of parasites in environmental samples, another factor that makes it difficult to detect these protozoa in such samples is the high concentration of the inhibitors of PCR enzymes, such as humic acid (MAHMOUDI et al., 2013). Researchers have demonstrated the reduction of inhibitory effects in PCR as well as the identification of parasites in samples with small amount of DNA by using chelating agents such as chelex-100 or probes to capture target DNA in the DNA extraction process, in addition to the use of bovine serum albumin in PCR (ANCENO et al., 2007; STROUP et al., 2012; KOKEN et al., 2013).

The use of guanidine thiocyanate followed by sonication showed efficacy in extracting DNA from oocysts; treatment with Chelex-100 after lysis was shown to be more effective in removing PCR inhibitors, compared to the inclusion of PCR facilitators during thermocycling (ANCENO et al., 2007). The use of probes to capture DNA, a method developed by Stroup et al. (2012), during DNA extraction from both protozoa showed improved sensitivity in the identification of these parasites in samples with less DNA, proving to be efficient in the detection of *Giardia* spp. and *Cryptosporidium* spp. in environmental samples.

Innovative techniques that do not use *Taq* DNA polymerase for DNA amplification, such as loop-mediated isothermal amplification (LAMP), which is characterized by isothermal amplification in turns and has become an useful diagnostic tool in parasitology with successful results in the identification of protozoans, including *Cryptosporidium* and *Giardia* spp. (BAKHEIT et al., 2008; PLUTZER; KARANIS, 2009; PLUTZER et al., 2010b; MAHMOUDI et al., 2013). A study by Mahmoudi et al. (2013) with water samples from Iranian rivers, demonstrated that LAMP is a good tool to complement PCR, because it is not affected by the presence of inhibitors, thereby

identifying positive samples that tested negative by conventional PCR.

Conclusion

The quality of water ingested by humans is of extreme importance in the prevention of gastrointestinal diseases caused by pathogens transmitted through the fecal-oral route, of which *Giardia* spp. and *Cryptosporidium* spp. are primarily responsible for waterborne outbreaks in the world. Factors related to the environmental phase characteristics of these parasites and the treatment process of water and sewage are important to explain the occurrence of disease in humans. However, several studies have showed that cattle are often infected by the subtypes and zoonotic genotypes of these protozoa, in addition to eliminating large amounts of oocysts in feces, and therefore, they play an important role in the contamination of environment and water resources. The epidemiological link between the humans infected with these protozoa and the ingestion of water contaminated with animal feces is still poorly explored in developing countries, in part because of less efficient diagnostic methods and lack of notification, and in part because of the non-molecular characterization of the identified protozoa, which is extremely important to establish the source of infection.

Detailed research and potability monitoring are warranted in rural areas that do not treat water for human consumption, and therefore, are more vulnerable to outbreaks. Molecular characterization of *Cryptosporidium* spp. and *Giardia* spp. identified in both water resources and animals and humans living in this area and epidemiological evaluation of the communities supplied by these resources allow improved understanding of the transmission cycle of these pathogens. In this way, it is possible to implement control and preventive measures to reduce the parasitic load in the environment and the

contamination of the water supply, thereby avoiding infections in humans and animals.

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