New insights into the detection and molecular characterization of Cryptosporidium with emphasis in Brazilian studies: a review

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ABSTRACT

Cryptosporidium spp. is a pathogenic protozoan present in the gastrointestinal tract of several hosts. This protozoan was originally classified as within the Coccidia Class and has recently been reclassified to gregarine based on studies that observed the evolutionary phases from the process of excision and sequencing of the 18S rRNA gene. Molecular biology techniques have become diagnostic tools and have also been used to understand the epidemiology of Cryptosporidium spp., since several species of this genus are very similar morphologically and morphometrically. Molecular techniques have been used in the identification of parasites, at the species and subtypes levels and to study disease transmission. The laboratory diagnosis of human cryptosporidiosis can be made by parasite detection methods, such as optical microscopy, antigens or genetic material detection, as well as serum antibodies raised to Cryptosporidium spp. Molecular methods were developed and allowed, not only an extensive revision of the taxonomy, but also an improvement in the laboratory diagnosis. In Brazil, there are few reports of Cryptosporidium spp. outbreaks in humans and all of them took place in nurseries. A few epidemiological studies developed in Brazil have used molecular methods for the detection of Cryptosporidium spp., as well as genotyping studies of their species and subtypes. The use of real-time PCR, together with microscopy and immunochromatography techniques, would result in a more precise diagnosis of cryptosporidiosis. The analysis of genotypes, subtypes and clonality of Cryptosporidium could be useful to understand and define the prognosis and severity of infections.

KEYWORDS: Cryptosporidium spp. Molecular characterization. Laboratory diagnosis. Epidemiological studies.

INTRODUCTION

Cryptosporidium spp. is a pathogenic protozoan present in the gastrointestinal tract of several hosts¹. This parasite was firstly described in 1907 by Ernest Edward Tyzzer, in the gastrointestinal epithelium of mice. Human infection was first described in 1976, in a child and in an adult in the same year. Cryptosporidium spp. has been a public health concern mainly due to reports of outbreaks in day care centers, immunosuppressed patients and also in waterborne transmission reports². Cryptosporidium spp. is one of the most prevalent waterborne parasites in the world and one of the main concerns in Public Health since the oocyst, the infecting form, is resistant to the most common disinfectants adopted in water treatment plants³,⁴. This protozoan was originally classified within the Coccidia Class⁵, but has recently been reclassified to gregarine based on studies⁶-⁸ that observed the evolutionary phases from the process of excision and sequencing of the 18S rRNA.
gene. *Cryptosporidium* spp. presents a morphological and phylogenetic relationship closer to gregarine than to coccidia. These findings were not adequately addressed by the scientific community until Clode *et al.* summarized them so that currently, *Cryptosporidium* spp. is classified in the sub-class *Cryptogregarina*, within the Class Gregarinomorpha, which is characterized as epicellular containing organisms, a food organelle very similar to that of the gregarines (epimerite) but it does not present apicoplast.

Another very relevant finding of *in vitro* and *in vivo* studies was the verification that *Cryptosporidium* spp. can develop its life cycle outside a host cell. The possibility of *Cryptosporidium* spp. multiplying in biofilms, in water or sewage treatment plants, led to new challenges for the correct management of these resources in view of a greater risk of outbreaks from waterborne transmission. In addition, microbial biofilms can form in the interior of the human intestine, increasing the host's susceptibility, as well as during the course of the infection.

Molecular biology techniques have become diagnostic tools that have been used to understand the epidemiology of many infectious agents, including *Cryptosporidium* spp., since several species of this genus are very similar morphologically and morphometrically. Molecular techniques have been used in the identification of parasites, at the species and subtypes levels, and in the study of disease transmission. This has had a great impact on epidemiological studies, taxonomy and classification of parasites, as well as on the understanding the pathogen's biology. According to Xiao *et al.*, with the application of molecular techniques in the taxonomy, it is possible to incorporate genetic data as one of the parameters to validate the *Cryptosporidium* species evaluation. Today, 38 species of *Cryptosporidium* have been described, going from amphibian parasites to mammals ones (Table 1), totaling more than 40 genotypes infecting mammals, but *Cryptosporidium parvum* and *Cryptosporidium hominis* are the two species predominantly reported in human infections. In the molecular characterization of the *Cryptosporidium* genotype, 18S rRNA genes are the most used because they present five copies per genome and are less polymorphic due to a slower evolution, therefore being the locus of choice for animal samples that may be infected by species or genotypes not yet classified.

Analysis of the DNA sequence encoding the GP60 glycoprotein is the most commonly used method to study *C. hominis* subtypes in humans and *C. parvum* subtypes in humans and ruminants. The gp60 gene (also called gp15/40) is similar to a microsatellite sequence because it has tandem repeats of TCA, TCG or TCT trinucleotides at the end of the gene. However, in addition to variations in the number of trinucleotide replications, there are extensive differences in sequences on regions without

### Table 1 - *Cryptosporidium* species* found in human infections.

| Species     | Main Host                   | Site of infection | Reference          |
|-------------|-----------------------------|-------------------|--------------------|
| *C. hominis*| Human                       | Small intestine   | Xiao*17            |
| *C. parvum* | Ruminants                   | Small intestine   | Tyzzer apud Xiao*17|
| *C. meleagris*| Turkey, Birds and human     | Small intestine   | Slavin*16          |
| *C. felis*  | Cats                        | Small intestine   | Xiao and Feng*67   |
| *C. canis*  | Dogs                        | Small intestine   | Fayer *et al.*18   |
| *C. ubiquitum*| Cattle, Ruminants, rodents, primates | Intestine | Fayer *et al.*1    |
| *C. muri*   | House mouse, Rodents        | Stomach           | Tyzzer apud Xiao*17|
| *C. viatorum*| Human                      | Small intestine   | Elwin *et al.*19   |
| *C. cuniculus*| European rabbits            | Intestine         | Robinson *et al.*20|
| *C. andersoni*| Cattle                    | Abomasum          | Lindsay *et al.*21 |
| *C. suis*   | Pigs                        | Small and large intestine | Ryan *et al.*22 |
| *C. bovis*  | Cattle                      | Small intestine   | Fayer *et al.*23   |
| *C. erinacei*| European hedgehog, horses   | Abomasum          | Kuć et al.*24      |
| *C. scrofarum*| Pig                       | Intestine         | Kuć et al.*25      |
| *C. tyzzeri*| Mouse, Rodents              | Small intestine   | Tyzzer*17; Ren *et al.*16 |
| *C. xiao*   | Sheep and goats             |                   | Fayer and Santín*17|
| *C. fayeri* | Kangaroo, Marsupials        | Small intestine   | Ryan *et al.*18    |

Adapted from: Xiao *et al.*14 and Ryan *et al.*15. *Chipmunk genotype I; Skunk genotype; Mink genotype; Horse genotype
repetitions that allow categorizing *C. parvum*, *C. hominis*, *Cryptosporidium ubiquitum*, *Cryptosporidium andersoni* and *Cryptosporidium meleagris* in families, according to the different subtypes. It is the marker with the largest polymorphism identified so far in the genome of *Cryptosporidium* spp.\(^{18-20}\). The *gp60* gene encodes a GP60 protein that is located on the surface of the apical region of invasive stages of the parasite and is one of the main targets of neutralizing antibody responses in humans\(^ {21}\). The distribution of families and subtypes of the *Cryptosporidium* species, mainly *C. parvum* and *C. hominis*, is still under investigation, with some results from different regions of the world. The following subtypes have been identified within *Cryptosporidium* species: 10 subtypes for *C. hominis* (Ia-Ik); 19 subtypes for *C. parvum* (Ia - It) and 10 subtypes for *C. meleagris*\(^{22-25}\). In relation to *C. hominis*, the Ib family is dominant in England, Portugal and Australia\(^ {26}\) and families Ia, Id and le are more common in Kenya, Malawi, India, Peru and the USA\(^ {16}\). In South Africa, the families most commonly found are If, Ib, Id\(^ {27}\), the If family has also been found in some samples from Portugal\(^ {26}\). Although *C. hominis* is considered a species of *Cryptosporidium* specific for humans, there are progressively more reports of this species in animals. *C. hominis* was detected in cattle\(^ {28}\), sheep and goats\(^ {29}\), kangaroos\(^ {30}\), rodents\(^ {31}\) and dogs\(^ {32}\), with the cosmopolitan subtype IbA10G2, associated with animal infection and being the dominant subtype in humans within these areas\(^ {33}\). In addition to the 18S rRNA and *gp60* gene targets, other genes have been used as tools in the search for the molecular characterization of *Cryptosporidium* species and subtypes, in line with several molecular techniques to detect these targets (Table 2).

In humans, a *Cryptosporidium* spp. infection may be asymptomatic, with mild clinical signs in the form of spontaneous diarrhea, or more severe symptoms with approximately 20 evacuations a day. The most serious clinical conditions are seen in immunocompromised individuals, including patients with immunological deficiencies related to malnutrition, patients undergoing chemotherapy, HIV-AIDS patients and transplant recipient patients\(^ {34,35}\). In healthy individuals, the incidence of cryptosporidiosis infections is higher in children and in the elderly due to the natural immunocompromising that exists in these age groups. In these individuals, cryptosporidiosis can turn into a chronic disease that lasts for months or even years. In AIDS patients, cryptosporidiosis can be extremely severe, with mortality rates as high as 50%. Additionally, cases of extra-intestinal cryptosporidiosis may occur, involving the hepatobiliary tract, lungs and pancreas. Biliary cryptosporidiosis is the most common manifestation after intestinal infection and has been described in 26% of AIDS patients\(^ {36}\). Respiratory tract infection is rare or may be underdiagnosed due to the lack of specific investigation. This infection is characterized by cough, dyspnea, fever and chest pain and may present radiological abnormalities similar to the ones of other pulmonary infections\(^ {37}\).

### Laboratory diagnosis

The laboratory diagnosis of human cryptosporidiosis can be performed by detection methods of the parasite such as optical microscopy, antigens or genetic material detection, as well as detection of antibodies against *Cryptosporidium* spp.

For a long time, the diagnosis of *Cryptosporidium* spp. was based only on the parasitological examination of stool samples (SPE), in which parasite oocysts were concentrated by flotation or centrifugation-sedimentation techniques. Immunological techniques for the detection of antigens in stool samples have also had a positive impact on diagnosis sensitivity using monoclonal antibodies in ELISA or Immunochromatographic assays\(^ {38}\). Serum antibody detection may also be performed, but this method has a lower diagnostic efficiency than antigen detection. The use of monoclonal antibodies has also significantly improved

| Table 2 - Molecular epidemiology and diagnosis tools for *Cryptosporidium* genotyping and subtyping. |
| --- |
| **Target** |
| ✓ SSU rRNA (18S rRNA gene) |
| ✓ 60-kDa glycoprotein gene (*gp60*) |
| ✓ 70-kDa heat shock protein gene (*hsp70*) |
| ✓ Oocyst wall protein gene (*cowp*) |
| ✓ Actin gene |
| ✓ β-tubulin gene |
| ✓ Thrombospondin-related adhesive protein gene (*trap-C1 ad trap-C2*) |
| ✓ ITS1 of rRNA |
| ✓ Dihydrofolate reductase gene (*dhfr*) |
| **Molecular techniques** |
| ✓ Single-round and nested PCR |
| ✓ PCR-RFLP |
| ✓ Real-time PCR |
| ✓ Single strand conformation polymorphism (SSCP) analysis |
| ✓ Melting curve analysis |
| ✓ Microarray |
| ✓ DNA sequencing |
| ✓ Multilocus fragment (MLFT) or sequence (MLST) typing |
| ✓ New generation sequencing (NGS) |

Adapted from unpublished data of Ana Luz Galvan.
the parasite detection in environmental samples, allowing the development of oocyst purification techniques such as paramagnetic beads conjugated to anti-Cryptosporidium monoclonal antibodies and fluorescence detection. However, these methods do not allow Cryptosporidium spp. species identification in samples. Molecular methods for the detection of parasite-specific DNA sequences were developed and allowed not only an extensive revision of the taxonomy, but also an improvement in the laboratory diagnosis, with definition of species and subtypes within these species in the samples.

**Stool parasitological examination**

The method of stool samples concentration for detection by optical microscopy aimed to increase the finding of parasites in these samples by examining density differences between different compounds present in feces, such as fats and debris, which can make visualization of the parasite difficult. The most common oocyst concentration techniques are flotation in saturated sucrose solution and sedimentation by means of ether-formalin and acetate-formalin gradients. For a better visualization of oocysts, light field or phase contrast optical microscopy are recommended. Oocysts are visualized as bright bodies containing black granules, when using phase contrast, while in a light field microscope, the oocysts appear translucent, often as slightly pinkish structures, containing granules in their interior. The simple centrifugation-flotation method, also called centrifugation-flotation in a hyper-saturated sucrose solution (Sheather’s solution), was developed in 1923 with the objective of promoting the identification of parasitic forms in animal feces. This technique uses a high density sucrose solution to promote the separation of protozoa cysts and coccidian oocysts, while using centrifugation as a way to promote the sedimentation of debris, forcing the fluctuation of parasitic structures so that they can be recovered later. In the centrifugation-flotation technique, another solute such as zinc sulphate can be used to promote the density gradient and fluctuation of parasitic evolutionary forms such as cysts and oocysts. Concentration techniques are always followed by staining methods, facilitating cysts observation. Staining techniques used in coccidia studies include the Kinyoun, Ziehl-Neelsen, negative staining, safranin, methylene blue, modified staining of Koster, Auramine, Nigrosin and Light Green. Other stains that can be used in coccidia research are Malachite Green, Giemsa, Auramine, Nigrosin and Light Green. However, these dyes do not have a well-known sensitivity and specificity, which makes it difficult to standardize the diagnostic routine for these protozoa in different clinical laboratories. The histopathological examination, with autopsy and biopsy materials, can also be routinely used, aiding in the identification of the parasite and allowing the observation of the parasite evolutionary forms.

For visualization of Cryptosporidium in tissues, the commonly used staining method is the hematoxylin-eosin in which the evolutionary forms are visualized as basophilic spherical bodies, from 2.0 to 7.5 μm, located on the surface of epithelial cells in histological sections. Other techniques that may be used are Schiff periodic acid and silver-based stains. Transmission electron microscopy allows the parasites’ ultrastructural morphology visualization.

Although Cryptosporidium spp. detection is primarily based on fresh or formalin-preserved fecal examination, the parasitological diagnosis has several limitations: it is time-consuming, requires an experienced microscopist to identify organisms and may exhibit limited sensitivity when there are few oocysts in fecal samples. In addition, this method is not useful for evaluating samples subjected to unfavourable conditions, such as frozen stool or environmental samples, which may modify the parasite morphology. Importantly, the microscopic methods do not allow the identification of species or genotypes involved in the infection.

**Immunological methods**

Immunological tests for antigen detection are simple, fast and specific. Despite having a higher cost, immunological tests require the use of monoclonal antibodies raised to specific targets for species differentiation and they present excellent sensitivity compared to the traditional staining techniques. The detection of antigens in feces (coproantigens) has become a useful tool in diagnosing cases of a suspected infection resulting from Entamoeba histolytica/Entamoeba dispar, Giardia lamblia and Cryptosporidium spp., whose fecal elimination usually
occurs intermittently. Immunological assays, such as the indirect immunofluorescence can also be used to identify *Cryptosporidium* spp. in water samples. In this case, variable volumes of water are required for analysis depending on the water samples type when using filtration followed by concentration, increasing the sensitivity of oocysts detection by fluorescence-labeled monoclonal antibodies and DAP (4′,6′-diamidino-2-phenylindole).

Detection of antibodies in serum samples from infected individuals can also be performed but presents problems due to difficulties in differentiating between a prior infection and a current infection, therefore decreasing the test value when samples from individuals in endemic areas with high parasite circulation are examined.

The identification of species through the production of antibodies against oocyst wall antigens is also hampered by the presence of conserved antigens among different species within the genus *Cryptosporidium*. To overcome this difficulty, molecular methods with sensitive and specific detection of DNA fragments from the parasite have been developed.

### Detection of genetic material

The detection of etiological agents genetic material has become a tool in the diagnosis of infectious diseases and to understand the epidemiology and taxonomy of etiological agents, including *Cryptosporidium* spp., characterizing different genotypes and subtypes. According to Smith, the taxonomy can incorporate genetic data as one of the parameters to validate *Cryptosporidium* species, with the application of molecular techniques.

The molecular methods currently used in the characterization of *Cryptosporidium* spp. are based on the polymerase chain reaction (PCR): nested-PCR, Single-Stranded Conformation Polymorphism PCR (PCR-SSCP), DNA probes hybridization, Restriction Fragment Length Polymorphism PCR (PCR-RFLP), multiplex-PCR and real-time PCR. These techniques are generally associated with sequencing of amplified fragments for results confirmation and to analyze the genetic diversity between species. These methods use several genetic markers, among them, the most reported in the literature are the small subunit rRNA (SSU) gene that encodes the smaller ribosomal subunit of 18S rRNA; the *cowp* gene, which encodes an oocyst wall protein; the *hsp70* gene, which encodes the heat shock proteins; ITS-1 and ITS-2, corresponding to internal transcribed spacer regions of ribosomal DNA; the *trap* gene (Thrombospondin-related adhesive protein of *Cryptosporidium*), and the gene encoding the GP60 or GP13/40 glycoproteins.

PCR-RFLP is a technique that consists of restriction enzymes or endonucleases to digest PCR products. These enzymes cleave the DNA at a specific site promoting its fragmentation in certain sizes so that the posterior analysis on agarose or polyacrylamide gel, resulting in different patterns according to the species of *Cryptosporidium* analyzed, allowing their identification. Xiao et al. standardized a protocol that differentiates almost all described species of *Cryptosporidium* using nested-PCR of the SSU rRNA gene and cleaving the products with the enzymes *SspI, VspI* and *Ddel*. The *Ddel* restriction enzyme differentiates *Cryptosporidium muris* from *C. andersoni*, while the first two differentiate *C. parvum, C. hominis, C. meleagris*, *Cryptosporidium canis* and *Cryptosporidium serpentis*, all belonging to the old group known as *C. parvum*. The use of PCR-RFLP with the restriction enzymes *SspI* and *VspI* is reported in at least 60% of the publications on the molecular epidemiology of cryptosporidiosis.

Real-time PCR is one of the most recent PCR method whose methodology employs fluorescent markers. Verweij *et al.* described a real-time multiplexing method for the detection of *Giardia lamblia, Entamoeba histolytica* and *C. parvum*. The results of this study demonstrated 100% specificity and sensitivity of the technique. The authors stated that real-time PCR, using probes, can reduce the risk of contamination, as well as processing time and cost of reagents due to the possibility of detecting different targets in the same assay. The technique developed by Ramirez *et al.* can diagnose the species of *C. hominis, C. parvum, C. canis, Cryptosporidium felis* and *C. meleagris* only using the melting curve. Melting points have a difference between species of at least 1 °C. The authors considered that the high specificity of the experiment is due to the small size (272 bp) of the amplified product, which lowers the melting temperature and increases the sensitivity of the test when compared to other protocols that amplify larger fragments. More recently, the real-time PCR method for detection of the genus *Cryptosporidium* and differentiation of *C. hominis* and *C. parvum* species using TaqMan probes from the 18S rRNA gene region was described and this methodology has also been used in our studies showing high efficiencies.
the TATATTT sequence in *C. parvum*. They observed that the sequences are rich in bases A and T. Years later, these results were confirmed in another phylogenetic study of *Cryptosporidium*\(^1\). They stated that the sequences of *C. hominis* and *C. parvum* showed differences in four regions of the 18S rRNA gene and that these differences were found in the first half of the gene sequence. The introduction of molecular tools has not only produced new insights into the epidemiology of cryptosporidiosis, but has also led to the identification of common modes of transmission such as zoonotic or anthroponotic transmission via the fecal-oral route and via contaminated food or water\(^1\).\(^4\).\(^6\).\(^8\).

**Cryptosporidium** spp infection in Brazil

In Brazil, there are few reports on *Cryptosporidium* spp. outbreaks in humans, all of them are in nurseries and most of them are in the Sao Paulo State\(^5\).\(^9\).\(^6\) (Figure 1). Usually, the cases are not properly documented as it is not an infection of obligatory notification in health services (Brazil Information System for Notifiable Diseases/Ministry of Health, Brazil). However, several studies have reported the presence of cysts and oocysts in water sources used for human supply\(^6\).\(^\text{3} \)-\(^\text{6}\).\(^\text{3}\). Although the origin of these outbreaks have not yet been identified, environmental contamination in different regions of the country has been described in several types of water sources such as surface and groundwater, treated water from natural sources, natural waters from mineral sources, raw sewage and treated, hospital sewage. For this reason, since 2000, the Brazilian Ministry of Health recommends the inclusion of *Cryptosporidium* spp. investigation in drinking water analysis\(^\text{64}\). Recently, monitoring *Cryptosporidium* oocysts and *Giardia* cysts in water collection points has become mandatory when the annual geometric mean of *Escherichia coli* exceeds 1,000 CFU/100 mL considering a minimum number of 24 samples collected uniformly for a minimum period of one year and maximum of two years. The obligation to maintain a systematic evaluation of water supply systems, from the perspective of health risks with the implementation of the Water Safety Plan has also been established\(^\text{65}\).

In a study carried out in Porto Alegre, Rio Grande do Sul State, the knowledge about this parasitic infection was evaluated among medical doctors. Of the 91 physicians interviewed, 83 (91.20%) admitted that they needed more information on the disease. This result suggests that the prevalence of cryptosporidiosis in Brazil is underestimated, since few laboratories of parasitology are concerned with a surveillance of this parasite by using routine techniques and they only perform tests upon medical request\(^\text{37}\).

In Brazil, studies on cryptosporidiosis carried out in the last two decades have shown heterogeneity in the selection of sample population, methods of conservation and analysis of fecal samples, as well as the choice of molecular characterization methods\(^2\). Using classical microscopy techniques, Mangini *et al*.\(^\text{66}\) studied parasitism by *Cryptosporidium* spp. in children with acute diarrhea in the city of Sao Paulo. From 1987 to 1990, 241 fecal samples

![Figure 1](image-url)  
**Figure 1** - Number of scientific articles published using detection methods and/or molecular characterization of *Cryptosporidium* in different regions of Brazil. (Data obtained until October 2018 in PubMed).
were examined, of which 17.43% revealed the presence of Cryptosporidium spp. oocysts, and infections occurred more frequently in children aged 6 to 12 months. In this study, as in other countries, a trend of seasonal variation in the occurrence of cryptosporidiosis was observed, since the frequency of children treated with diarrhea was higher between March and May. In the municipality of Uberlandia, Minas Gerais State, the frequency of oocysts of Cryptosporidium spp. in diarrheal stools of children was 4.26% in a total of 94 samples. The report of Agnew et al. performed a genotyping study of Cryptosporidium spp., associated with a diarrhea outbreak in Sao Paulo, Sao Paulo State, using the sequence of three different locus (SSU rRNA, covp gene and ML1 microsatellite) and observed through the ML1 results that C. hominis was the only genotype identified in the 29 analyzed samples. The molecular analysis reinforced the hypothesis that the transmission of C. hominis during the outbreak of diarrhea occurred through person-to-person transmission, fecal-oral route. Also, in Sao Paulo, two other studies carried out the molecular characterization of Cryptosporidium spp. isolates, and C. hominis was the dominant species, but C. parvum, C. felis, C. meleagridis and C. canis were also found. Rolando et al. conducted a survey on fecal samples collected from 1999 to 2010 in the city of Rio de Janeiro, searching for Cryptosporidium spp. using optical microscopy and real-time PCR, which can detect the genus Cryptosporidium and differentiate between C. hominis and C. parvum. From a total of 1,207 samples, 48 (3.98%) were positive for Cryptosporidium spp. based on light microscopy results and amplification by a real-time PCR with a genus-specific probe. Of these 48 samples, 37 (70.78%) showed amplification for C. hominis and 5 (10.42%) showed amplification for C. parvum. The six (12.50%) samples that amplified only the genus, probably belonged to other species also found in human infections.

Peralta et al. aimed to better understand the main species present in clinical specimens from three public hospitals in Rio de Janeiro, Rio de Janeiro State. The group worked with 90 samples from patients of these hospitals who presented a requisition for coccidia investigation. All samples, independent of microscopy results, were analyzed by real-time PCR. Of these samples, 19.1% were positive for Cryptosporidium spp. under microscopy, while real-time PCR showed amplification for Cryptosporidium spp. in 4.5% of the samples, for C. hominis in 15.7% of the samples and for C. parvum in 4.5% of the samples. Amplification was observed for these two species in a single sample. In these samples, nested-PCR for the 18S rRNA and nested-PCR for gp60 were also carried out. Only 22.2% of the samples amplified for the 18S rRNA gene, but there was agreement with the real-time PCR in the definition of species, and 72% of the samples amplified for the gp60 gene. Analysis
of the gp60 gene fragment identified the subtype IbA10G2 in all samples of C. hominis and the subtype IcA5G3 (anthroponotic nature) for the samples with C. parvum. According to some authors, these subtypes are cosmopolitan and the IbA10G2 is considered the most virulent.\textsuperscript{14,16,33,79,80}

The studies with environmental and animal samples are more advanced, as some studies performed the molecular characterization of species and sub-types of Cryptosporidium in both, water and soil samples, as well as in farm animals, birds, captive animals, reptiles, etc. These studies represent a major advance in the understanding of the anthropozoonotic transmission and for the development of preventive strategies to avoid environmental and human contamination. Cryptosporidiosis has a wide distribution in animals (farmed animals, confined animals, wild animals, etc.) based on observations of cryptosporidiosis reported in the literature.\textsuperscript{59,81-83}

The main source of this parasite in the environment is the spread of feces from various animals. According to Graczyc \textit{et al.},\textsuperscript{84} migratory birds fly in flocks and travel long distances, contributing to the environmental dissemination of infectious agents. Significant evidence indicates that birds play an important role in the transmission cycle of parasites, since in addition to migrating, they also reproduce and defecate in water sources and can contaminate important reservoirs.\textsuperscript{84,85} However, the studies performed in Brazil, regarding the presence of Cryptosporidium spp. in animals kept in captivity, are case reports with genus identification of the protozoan, and a few reports on the genotypes and subtypes, making it impossible to infer on zoonotic transmission possibilities, identification of the sources of infection or the epidemiology of the parasite in these environments. The same is true in free-living animals in both, wild and urban environments, as well as pet stores and animals that are kept in wildlife centers and zoos.

The lack of information on domestic animals is troubling, especially for the understanding of the relationship between animal and human contamination and what is interfering with the environment. These studies may provide information regarding the infectivity, as well as the virulence of the parasite. Therefore, future research is necessary to determine the susceptibility of different groups of hosts to Cryptosporidium spp in Brazil.

The diagnosis of cryptosporidiosis has been showing high sensitivity and specificity, at a moderate cost. This is a reality, but adequate political reforms must be implemented to allow access to a precise result, especially in patients in whom the differential diagnosis is unpredictable for the maintenance of life. The use of real-time PCR, together with microscopy and immunochromatography techniques, would result in a more precise diagnosis of cryptosporidiosis. The analysis of genotypes, subtypes and clonality of Cryptosporidium could be helpful to understand and define the prognosis and severity of the infection.

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\section*{CONFLICT OF INTERESTS}

The authors have no conflict of interests in this study.

\section*{AUTHORS’ CONTRIBUTIONS}

All authors prepared the bibliographic survey, manuscript preparation and approved the final version of the manuscript.

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