Genotypic Characterization of *Cryptosporidium hominis* from Water Samples in São Paulo, Brazil

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Abstract. The protozoan parasite *Cryptosporidium* has emerged as one of the most important water contaminants, causing waterborne outbreaks of diarrheal diseases worldwide. The small size of oocysts under the microscope and the possibility of changes in characteristics of oocysts, mainly in environmental samples, make the taxonomy of the genus difficult if morphologic characteristics are considered. This limitation encouraged the application of molecular methods to identify this microorganism. The aim of this study was to detect and identify by nested-polymerase chain reaction oocysts of *Cryptosporidium* present in water samples in the state of São Paulo, Brazil. Water samples were concentrated through a membrane filter, DNA was extracted by using a standard technique, and both amplification reactions used forward and reverse oligonucleotides that were complementary to *Cryptosporidium* 18S ribosomal RNA gene sequences. Thirty water samples from different sites of collection in the state of São Paulo were evaluated. *Cryptosporidium* oocysts were detected in 30% of the samples. By genotyping, *C. hominis* and *Cryptosporidium* sp. were identified in recreational water and *C. meleagridis* was identified in surface water samples. This is the first report of *C. hominis* in environmental samples in Brazil. Although identification of *Cryptosporidium* is still a difficult task, molecular methods are essential for specific identification and are a helpful tool to aid to understand the epidemiology of this parasite in Brazil.

INTRODUCTION

The protozoan parasite *Cryptosporidium* has emerged as one of the most important contaminants of water, causing waterborne outbreaks of diarrheal diseases worldwide. Several outbreaks have been reported in which the source of oocysts were the aquatic environment, including surface and recreational waters.¹ The most important waterborne outbreak of *Cryptosporidium* occurred in Milwaukee, Wisconsin, in 1993 and affected approximately 403,000 persons. Since that time, this protozoan is considered one of the main pathogens responsible for gastrointestinal disease associated to water transmission in the United States.²

Cryptosporidium is a strict intracellular parasite, and its infectious forms are oocysts eliminated in stool of several hosts, including humans.³ Domestic and wild animals are important reservoirs in the transmission of this zoonosis to humans, and water plays an important role in the spread of contaminant oocysts, which are excreted in a variety of environments.^{4,5}

The oocysts can survive for long periods in fresh waters, and are resistant to water chlorination processes. Another contributing factor that makes it difficult to remove oocysts is their small size, 3–7 µm, which decreases the elimination capacity of filtration processes.^{4,6}

Correct identification of this pathogen is extremely important, not only because of clinical aspects, but also for epidemiologic studies. Although conventional methods used to identify parasite agents in stool samples show good results and applicability, these methods could represent a complex task when environmental samples are considered.

Several molecular techniques have been recently developed to detect nucleic acid polymorphism or allelic variation at

enzymatic levels in different classes of microorganisms. These tools have increased knowledge of the genetic structure, differentiation, and classification of *Cryptosporidium* species.⁷

Currently, the literature reports several methods for detection, identification, and enumeration of *Cryptosporidium* oocysts in water and fecal samples. Therefore, standardization of efficient, reproducible, and simple techniques are still needed to ensure that these methods will not remain restricted to research centers.⁸

In São Paulo State, Brazil, studies have described the presence of *Cryptosporidium* oocysts in water sources,^{9–12} sewage,^{13,14} bottled mineral water,¹⁵ marine bivalve mollusks,¹⁶ and day care centers,^{17,18} but no waterborne infections have been reported.

Only a few studies in Brazil have applied molecular methods to detect *Cryptosporidium* oocysts in fecal samples.^{19,20} To our knowledge there are no available studies using molecular methods for detecting and genotyping these protozoan in surface and recreational waters in Brazil. However, *Cryptosporidium* in domestic and wild animals in Brazil has been investigated, mostly because of their importance as reservoirs and in spread of the parasite (Table 1).

The aim of the present study was to conduct a preliminary investigation on the presence of *Cryptosporidium* species in water sources in the State of São Paulo, Brazil, to understand the distribution and dissemination route of this pathogenic protozoan parasite.

MATERIALS AND METHODS

Sampling collection and preparation. Thirty water samples were examined during May 2005–December 2006. Twelve of these samples were recreational water obtained from the Ribeirão da Fazenda Basin on the northern coast of São Sebastião, São Paulo State. The remaining 18 samples were surface water obtained from locations at the Water Resource Management Unities (UGRHI) located around São Paulo: UGRHI-02 Paraíba do Sul River Basin, UGRHI-05 Piracicaba

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TABLE 1
Studies applying molecular methods for identification of *Cryptosporidium*, São Paulo, Brazil*

Molecular method	Source of fecal samples	Species	Reference
Nested PCR and DNA sequencing	Birds	<i>C. baileyi</i> , <i>C. galli</i> , <i>C. parvum</i> , <i>C. meleagridis</i> , and <i>C. avian</i> genotypes I, II, III	46
Nested PCR and DNA sequencing	Lambs	<i>C. parvum</i> type A, <i>C. parvum</i> type B, and cervid genotype	48
Nested PCR and DNA sequencing	Birds	<i>C. galli</i>	41
Nested PCR and RFLP	Humans	<i>C. hominis</i> and <i>C. parvum</i>	20
Nested PCR and RFLP	Humans	<i>C. hominis</i> , <i>C. parvum</i> , and <i>C. meleagridis</i>	40
PCR, RFLP and DNA sequencing	Domestic animals	<i>C. baileyi</i> , <i>C. meleagridis</i> , <i>Cryptosporidium</i> sp., <i>C. felis</i> , <i>C. canis</i> , and <i>C. parvum</i>	39
Nested PCR and DNA sequencing	Capybara (<i>Hydrochoerus hydrochaeris</i>)	<i>C. parvum</i> type A and <i>C. parvum</i> subtype IiaA15G2R1	45
PCR and RFLP	Humans	<i>C. hominis</i> and <i>C. parvum</i>	49
PCR	Canines	<i>C. parvum</i>	43
Nested PCR and DNA sequencing	Ostriches (<i>Struthio camelus</i>)	<i>C. baileyi</i> isolate ostrich BR-01	44
PCR and DNA sequencing	Humans	<i>C. hominis</i>	18
Nested PCR and RFLP	Ostriches (<i>Struthio camelus</i>)	<i>C. baileyi</i> and <i>C. meleagridis</i>	47
Nested PCR and RFLP	Humans	<i>C. parvum</i>	42

* PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism.

Basin, Capivari and Jundiá River Basin, UGRHI-06 Alto Tiete Basin, and UGRHI-07 Baixada Santista (Table 2).²¹

Samples (10 liters) were collected and concentrated within 24 hours of collection by using a modified membrane filtration technique. After centrifugation, the pellet was stored at -20°C for further processing. Extraction of genomic DNA was performed by using the phenol-chloroform-isoamyl alcohol protocol, and DNA was stored at 4°C. Nested polymerase chain reaction (PCR) assays were performed using primers specific for a polymorphic region of the 18S ribosomal RNA gene of *Cryptosporidium* spp. The initial PCR was performed with primers SCL1 and CPB-DIAGR, which amplified a fragment of 1,035 basepairs. For the second amplification, primers used were SCL2 and SCR2, which amplified a fragment of approximately 220 base pairs. The nested PCR product was visualized by electrophoresis on a 2% agarose gel and staining with ethidium bromide.²²⁻²⁴

Sequencing and phylogenetic analysis. Molecular cloning and sequencing were carried out as described by Ivanova and others.²⁵ Analyses of 220 positions of 18S ribosomal RNA gene sequences of the clones were compared with their closest relatives in GenBank by BLAST searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Additional phylogenetic and molecular evolutionary analyses were performed with MEGA version 4.1 software^{26,27} and the neighbor-joining method.^{28,29}

GenBank accession numbers. GenBank accession numbers of 18S ribosomal RNA gene reference sequences used in this study for alignments and phylogenetic tree construction were AF108865 for *C. hominis*, AF115378.1 for *C. wrairi*, AF112574.1 for *C. meleagridis*, AF112575.1 for *C. felis*, AF112573.1 for *C. saurophilum*, AF093493.1 for *C. parvum*, AF108860 for *C. fayerii*, AB210854.1 for *C. canis*, AF108861 for *C. suis*, EF514234.1 for *C. bovis*, AY954882.1 for *C. baileyi*, AF093502.1 for *C. serpentis*, L19069.1 for *C. muris*, and AY954885.1 for *C. andersoni*. Sequences E440, E456, E508, and E441 refer to clinical samples from São Paulo, Brazil.

RESULTS

In the present study, 30 water samples from several sites in the State of São Paulo were collected during 2005–2006, of which 9 were positive for *Cryptosporidium* by PCR (Table 2). At one of the collection sites (Ribeirão da Fazenda, São

Sebastião), 12 recreational water samples were collected, of which 5 (41.7%) were positive. For the superficial water samples collected from rivers and reservoirs (Piracicaba/Piracicaba, Piracicaba/Americana, Atibaia/Atibaia, and Guarapiranga Dam), molecular detection was possible in 4 (22.2%). Results obtained in this study demonstrate the difficulty in detection and identification of *Cryptosporidium* in environmental samples already reported in previous studies.

DISCUSSION

Our study is the first in Brazil in which genotyped *Cryptosporidium* oocysts were detected in water samples. Although the PCR is extremely sensitive and efficient when a pure DNA suspension is used, amplification can be reduced by inhibitor components frequently found in stool and environmental samples. Several studies used replicas in PCR assays when working with water samples, not only to increase the number of oocysts, but to facilitate identification of multiple species that can be present in this type of sample.^{24,30-32}

In Brazil, several studies have reported *Cryptosporidium* oocysts in raw or treated sewage and bottled mineral water by using immunomagnetic separation or Percoll or sucrose gradients, followed by immunofluorescence, phase contrast microscopy, or epifluorescence.^{13-15,33}

Hachich and others¹¹ studied different hydrographic basins containing water for human consumption in the State of São Paulo by using the 1623 USEPA method. They reported prevalences of 2.5% for *Cryptosporidium* oocysts and 27% for *Giardia* cysts. In a study conducted by the Company of Environmental Sanitation Technology in São Paulo, eight water resources management units in specific areas of the State of São Paulo surrounded by highly populated and industrialized areas were tested for *Cryptosporidium* and *Giardia*. Results of this study showed that of 207 water samples analyzed by using the 1623 USEPA method, 8.7% were positive for *Cryptosporidium*.³⁴ In addition, inter-laboratory assays have been performed to evaluate comparability and reproducibility of standardized molecular tools used to genotype *Cryptosporidium*.³⁵⁻³⁷

Because of the low number of oocysts present in water samples, mainly in surface water in which they are diluted, large volumes of water are required to perform the assays.

TABLE 2

Positive and negative results for genotyping of *Cryptosporidium* in the studied samples, São Paulo, Brazil*

Sample	Collection sites	Species	PCR result
1		—	P
2		—	P
3		—	N
4		—	N
5		<i>C. hominis</i>	P
6	Ribeirão da Fazenda S. Sebastião/SP Channel Pontal da Cruz	—	N
7		—	N
8		—	N
9		—	N
10		—	N
11		—	P
12		<i>Cryptosporidium</i> sp.	P
13	Piracicaba River, Piracicaba (UGRHI 05)	—	P
14	Cotia River, ETA† from down Cotia (UGRHI06)	—	N
15	Piracicaba River, Americana (UGRHI 05)	—	P
16	Parafba River, Aparecida (UGRHI 02)	—	N
17	Guarapiranga Reservoir – SABESP (UGRHI 06)	<i>C. meleagridis</i>	P
18	Atibaia River, Atibaia (UGRHI 05)	—	N
19	Atibaia River, Campinas (UGRHI 05)	—	P
20	Piracicaba River, Americana-Cariobá (UGRHI05)	—	N
21	Atibaia River, Atibaia (UGRHI 05)	—	N
22	Atibaia River, Campinas-Valinhos (UGRHI 05)	—	N
23	Piracicaba River, Americana-Cariobá (UGRHI 05)	—	N
24	Capivarí River, Campinas (UGRHI 05)	—	N
25	Atibaia River, Atibaia (UGRHI 05)	—	N
26	Atibaia River, Campinas-Valinhos (UGRHI 05)	—	N
27	Cotia River, ETA† from down Cotia (UGRHI 06)	—	N
28	Parafba River, Aparecida (UGRHI 02)	—	N
29	Parafba River, Aparecida (UGRHI 02)	—	N
30	Exit Channel II, H. Borden Usine (UGRHI 07)	—	N

* PCR = polymerase chain reaction; P = positive; N = negative; UGRHI = water resource management unities.

† Water treatment station.

In addition to the USEPA 1623 method,³⁸ other methods such as standard methods for examination of water and wastewater and the alternate method have been used worldwide.⁸ These methods require filtration through filter membranes or cartridges, sample purification, and immunofluorescence visualization by using specific antibodies.⁸

In Brazil, data on genotypic identification of *Cryptosporidium* from environmental samples are scarce. However, in our study, analysis of *Cryptosporidium* 18S ribosomal RNA gene sequences obtained by the neighbor-joining method identified *C. hominis* and a *Cryptosporidium* sp. in recreational water samples and *C. meleagridis* in superficial water from the

Guarapiranga Dam (Figure 1). Therefore, species circulating in water in Brazil may be underestimated.

Sequencing of samples in our study resulted in profiles compatible to the species *C. hominis* (EU853174), *C. meleagridis* (EU853175.1), and *Cryptosporidium* sp. (EU853176) when compared with homologous sequences available in GenBank. Among the species identified, *C. hominis* showed 100% similarity with sequences in GenBank (Figure 1).

Some characteristics of the sites where each sample was collected may have been favorable for *C. hominis*. On the basis of the data in this report, the presence of *Cryptosporidium* species indicates that the most probable source of contamination is human. Recreational water sites (Ribeirão da Fazenda River) often show contamination because of human activities, which represents a risk factor for enteric diseases. This contamination decreases water quality.

Recently, *C. meleagridis*, *C. bailey*, *C. felis*, *C. canis*, and *C. parvum* were identified in fecal samples from a variety of domestic animals in Rio de Janeiro, a neighbor state of São Paulo.³⁹ In our study, *C. meleagridis* was identified in sample 17 from the Guarapiranga Dam (Table 2). This species was identified as the etiologic agent of diarrhea in domestic birds, and its zoonotic characteristics are well elucidated. This species is now recognized as the third most common *Cryptosporidium* species in humans. This species has also been detected in immunocompromized patients in Brazil, which makes the results obtained in this study of great epidemiologic importance.⁴⁰

For any surveillance system, it is important to know which species of pathogenic microorganisms are infecting humans and what are the sources of infection. The use of genotyping methods to identify *Cryptosporidium* oocysts of clinical interest for humans present in environmental samples would provide valuable information to sanitary authorities. This information will be helpful in prevention and control of cryptosporidiosis. The results of this study suggest that the method used for

FIGURE 1. Phylogenetic relationship among *Cryptosporidium* sequences retrieved from GenBank and the sequences EU853174, EU853175 and EU853176 genotyped in this study, inferred by maximum parsimony likelihood analysis of the partial 18S ribosomal RNA gene. Values on branches represent bootstrapping using 1,000 replicas.

concentration and detection of DNA from *Cryptosporidium* species was sensitive and specific, even at low concentrations of oocysts in water samples.

The availability of drinking water and adequate sanitation are essential for maintenance of human and environmental health. However, if one considers the importance of *Cryptosporidium* as a waterborne agent and the low infectious dose (1–100 oocysts), it is extremely important that appropriate treatment of water sources used for human consumption is conducted.

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