

# Genotypic characterization and phylogenetic analysis of *Cryptosporidium* sp. from domestic animals in Brazil

F. Huber<sup>a,\*</sup>, S. da Silva<sup>b</sup>, T.C.B. Bomfim<sup>c</sup>,  
K.R.S. Teixeira<sup>d</sup>, A.R. Bello<sup>b</sup>

<sup>a</sup> Instituto Superior de Tecnologia/ Fundação de Apoio à Escola Técnica do Estado do Rio de Janeiro (FAETEC),  
Rua João de Lacerda S/N, Bairro Fábrica, Paracambi, RJ, CEP 26600-000, Brazil

<sup>b</sup> Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Do Estado do Rio de Janeiro,  
Av. Professor Manuel de Abreu, 444, Rio de Janeiro, RJ, CEP 20550-170, Brazil

<sup>c</sup> Universidade Federal Rural do Rio de Janeiro, Curso de pós-graduação em Ciências Veterinárias,  
Departamento de Parasitologia Veterinária, BR 465, Km 7, Seropédica, RJ, CEP 23890-000, Brazil

<sup>d</sup> Laboratório de Genética e Bioquímica, Embrapa Agrobiologia, Rodovia BR 465, Km 7,  
Seropédica, RJ, CEP 23890-000, Brazil

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## Abstract

The purpose of the present study was the genetic characterization, sequencing and phylogenetic analysis of 18S rDNA sequences of *Cryptosporidium* isolates obtained from different animal hosts in Brazil. Fecal samples containing *Cryptosporidium* oocysts were obtained from chickens, ducks, quails, guinea pigs, dairy calves, dogs and cats. For amplification of 18S rDNA sequences the Secondary-PCR product of the extracted DNA from fecal suspension of each studied animal was utilized. The primary genetic characterization of *Cryptosporidium* sp. was performed using RFLP with the enzymes *SspI* and *VspI*. DNA samples were sequenced and subjected to phylogenetic analysis. The results showed *C. baileyi* infecting two ducks and one quail and *C. meleagridis* infecting one chicken. The sequences obtained from *Cryptosporidium* sp. infecting guinea pigs were not identified within groups of known *Cryptosporidium* species. The isolates found parasitizing cats and one dog were diagnosed as *C. felis* and *C. canis*, respectively. One isolate of calf origin was identified as *C. parvum*. The phylogenetic analysis showed clear distribution of isolates between two *Cryptosporidium* sp. groups according to their gastric or intestinal parasitism. A great genetic distance was observed between *C. felis* and *C. canis* from Brazil when compared to the reference sequences obtained from GenBank. The results obtained during this study constitute the first report of rDNA sequences from *C. baileyi*, *C. meleagridis*, *C. felis*, *C. canis* and *C. parvum* isolated in Brazil. © 2007 Elsevier B.V. All rights reserved.

**Keywords:** *Cryptosporidium*; Sequences; Domestic animals; Brazil

## 1. Introduction

The genus *Cryptosporidium* belongs to the phylum Apicomplexa, and these organisms parasitize the intestinal tract of a great range of vertebrates, including

humans. Although Tyzzer first described the genus in 1910, its pathogenicity was reported only in 1955 by Slavin, who associated the infection of *C. meleagridis* with the mortality of young turkeys on a commercial farm.

The transmission of the parasite occurs directly by the fecal-oral route, via ingestion of infective oocysts. In humans and in other mammals, *C. parvum* is known to be a serious pathogen causing acute diarrhea, affecting

\* Corresponding author. Tel.: +55 21 2682 1617.

E-mail address: [franziska.huber@gmail.com](mailto:franziska.huber@gmail.com) (F. Huber).

mostly children and individuals with deficient immunological systems (Abe et al., 2002). Other species of *Cryptosporidium* have also been reported and the zoonotic transmission is dependent on the species of *Cryptosporidium* involved and the susceptibility of the host.

The genus *Cryptosporidium* comprises 15 species. *C. muris* (Tyzzer, 1907); *C. parvum* (Tyzzer, 1912); *C. wrairi* (Vetterling et al., 1971); *C. felis* (Iseki, 1979); *C. andersoni* (Lindsay et al., 2000); *C. canis* (Fayer et al., 2001); *C. hominis* (Morgan-Ryan et al., 2002); *C. suis* (Ryan et al., 2004) and *C. bovis* (Fayer et al., 2005) infecting mammals. Infecting birds were described *C. meleagridis* (Slavin, 1955); *C. baileyi* (Current et al., 1986) and *C. galli* (Pavlásek, 2001). Infections in reptiles are related to *C. serpentis* (Levine, 1980) and *C. saurophilum* (Koudela and Modrý, 1998) while *C. molnari* (Alvarez-Pellitero and Sitjá-Bobadilla, 2002) to infections in fish.

In domestic animals, *C. felis* is found to infect primarily cats while *C. canis* infects dogs (Morgan et al., 2000; Fayer et al., 2001). The occurrence of *C. parvum*, *C. andersoni* and *C. bovis* has been reported in ruminants (Fayer et al., 2005; Xiao et al., 2004). In wild and domestic birds cryptosporidiosis is associated with *C. galli*, *C. baileyi* and *C. meleagridis* (Ryan et al., 2003).

Most of these species have already been diagnosed in hosts other than those usually described. There are some registered cases of *C. felis* infecting humans and even a cow (Bornay-Llinares et al., 1999; Pieniazek et al., 1999; Xiao et al., 2001; Cama et al., 2003). During experimental infection studies, *C. parvum* was able to infect cats and dogs (Fayer et al., 1997; Darabus and Olariu, 2003) and in natural infections, *C. canis* was also observed to infect humans (Xiao et al., 2001; Cama et al., 2003). In recent years researchers' attention has been directed at the ability of *C. meleagridis* to infect humans, this species being considered the third most diagnosed in this host (Xiao et al., 2001, 2004; Coupe et al., 2005). Despite these cases of human infection with animal-related species of *Cryptosporidium*, the most common species found in humans are *C. hominis* and *C. parvum* (Peng et al., 1997).

Since, in general, all *Cryptosporidium* species are morphologically very similar and have low host specificity, molecular studies have become essential for the correct identification of the species involved in outbreaks and epidemiological studies. For the naming and validation of *Cryptosporidium* sp., morphological measurements, biological data, including cross-transmission studies, and molecular data should be associated (Xiao et al., 2000b)

The genus *Cryptosporidium* has been intensively studied by several groups in Brazil. However, to date no information on genetic sequences of isolates from *Cryptosporidium* sp. associated with animal hosts in Brazil have been reported.

The objectives of the present study were the genotypic characterization, sequencing and phylogenetic analysis of Brazilian isolates of *Cryptosporidium* sp. of different animal host origins. To achieve this, species of *Cryptosporidium* causing natural infections in animals sold at a public market, in cats and dogs from an animal shelter, and in calves from a dairy farm were studied.

## 2. Materials and methods

### 2.1. Sites of animal origin, fecal sampling, and isolation of oocysts for analysis

#### 2.1.1. Public market

The indoor public market, located in Rio de Janeiro city, contained 50 shops and stands offering a great variety of products, included two animal trading stores offering birds (ducks, chickens, quails and canaries) and small mammals (hamsters, rabbits and Guinea pigs). These shops were located between stands selling products and meat for human consumption. Contact between customers and animals were not hampered by any kind of barrier and any customer could touch the animals.

The animals were housed in separate stacked-up cages, with removable trays for cleaning. Canaries and pigeons occupied the two top cages, and the three cages below held ducks, chickens and quails aged two to seven days. The two cages located at the very bottom held guinea pigs and rabbits.

To verify the occurrence of *Cryptosporidium* sp. in the animals, samples of feces were collected from the individual cages and examined.

Animals originally held in cages found contaminated by oocysts were acquired 24 h after the first sample analysis. In the laboratory, the animals were held in individual cages, receiving food and water *ad libitum*. Daily fecal samples were collected and examined as described below.

#### 2.1.2. Cat shelter

Thirty fecal samples were collected from cats kept at an animal shelter in Nova Iguaçu (Rio de Janeiro State). The cats were originally strays, found and taken to the shelter, where they were kept in two enclosures holding a total of 80 cats.

Fecal samples were collected randomly directly from the floor, taking care to collect only fresh feces that had not been stepped on by other animals. Fecal samples from four puppies in the shelter were also collected. They were of unknown breed, and were kept outside the cat enclosures along with their mother.

### 2.1.3. Calves from a dairy farm

Fecal samples from 56 dairy calves were collected directly from the rectum of the animals at a dairy farm, located in Rio de Janeiro City. The calves were 1–14 days old and had been separated from their mothers just after birth. They were bottle fed with colostrum.

### 2.1.4. Processing of fecal samples

The fecal samples were placed on ice and taken to the Laboratory for processing.

Copro-parasitologic examinations were conducted as described below (Huber et al., 2005), using the centrifuge-floatation technique. The quantity of feces processed in each exam varied between 1 and 2 g, except the samples from the birds, in which case less than 1 g of feces was examined from each sample.

In the laboratory, the samples were registered, homogenized with 30 ml of distilled water, thereafter filtered through fine plastic disposable sieves with an overlay of gauze. After this the fecal material was divided in two conical 15 ml test tubes and centrifuged at  $402.48 \times g$  for 10 min. The supernatant was discarded and the precipitate was suspended in saturated sugar solution (density of 1.30 g/ml) and centrifuged  $402.48 \times g$  for 5 min. The tube was filled up with sugar solution, covered with a slide cover slip and left at rest for three minutes. The cover slip was mounted on a glass slide and examined under an Olympus BX 51 binocular microscope at  $40\times$ , with phase contrast, using the  $100\times$  objective to confirm visualization of the protozoans. Beside *Cryptosporidium*, the presence of other parasite oocysts, cysts or eggs was evaluated. In cases where more than four *Cryptosporidium* oocysts were visualized, the sediment of the second tube was utilized for DNA extraction and subsequent PCR detection.

## 2.2. Total DNA extraction from the fecal samples

A 200  $\mu$ l aliquot of the former described precipitate was used for extraction. The extraction protocol was based on that proposed by McLauchlin et al. (1999), Zhu et al. (1998) and Boom et al. (1990), with some

modifications. In a 1.5-ml Eppendorf microtube were added 200  $\mu$ l of fecal sample 500  $\mu$ l of DNAzol<sup>®</sup> (Invitrogen), 0.5% (final concentration) of polyvinyl-pyrrolidone (PVP, Sigma) and approximately 0.2 g of glass beads of 425–600  $\mu$ m diameter (Sigma). The microtubes were vortexed three times and incubated at 96 °C for 60 min.

After centrifugation, the supernatant was transferred to another microtube, precipitated with 1 ml of pure ethanol and centrifuged again. The supernatant was discarded and the pellet washed twice with 500  $\mu$ l ethanol (95%). The ethanol was discarded and the pellet re-suspended in 500  $\mu$ l DNAzol, and after centrifugation the supernatant was transferred to a new microtube. The DNA precipitation and washing steps were then repeated as described above. The resulting pellet was suspended in 100  $\mu$ l TE and then the DNA solutions were used for further analysis or stored at –20 °C before analysis.

### 2.2.1. PCR detection

For the primary PCR (expected amplicon size: 1325 bp), primers were used as described by Xiao et al. (2004): 18 SF: 5'-TTCTAGAGCTAATACATGCG-3' (forward) and 18 SR: 5'-CCCATTTCC TTCGAAA-CAGGA-3' (reverse). For the secondary PCR (expected amplicon size: 819–825 bp, depending on the species), the following primers were used: 18 SNF: 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' (forward) and 18 SNR 5'-AAGG AGTAAGGAACAACCTCCA-3' (reverse).

The primary polymerase chain reaction (PCR) contained: 4 mM of MgCl<sub>2</sub>, 0.2  $\mu$ M of each primer (18SF and 18SR),  $1\times$  Taq buffer, 200  $\mu$ M of each deoxyribonucleotide, 2.5 U of Taq DNA polymerase (Cenbiot<sup>®</sup>), 2  $\mu$ l of DNA sample and milliQ water up to a total volume of 50  $\mu$ l.

The conditions for the secondary PCR were the same as for the primary PCR, except the use of a different pair of primers and of 3 mM of MgCl<sub>2</sub>.

Both procedures were performed in a thermocycler (Eppendorf<sup>®</sup>) with the following cycles: 35 cycles of 94 °C/45 s, 55 °C/45 s, 72 °C/1 min, with a final extension of 72 °C/7 min.

## 2.3. RFLP analysis of the secondary PCR products with restriction enzymes

In order to identify the *Cryptosporidium* species, the digestion of 10  $\mu$ l of the secondary PCR products using *SspI* enzyme (Invitrogen) was performed according to the manufacturer's recommendations, except that four

enzyme units were used in each reaction. The final volume of each reaction was 50  $\mu$ l.

For samples presenting similar restriction pattern with *SspI*, as occurs with *C. parvum*, *C. canis* and *C. meleagridis* a second RFLP analysis was performed with *VspI* enzyme.

Following the digestion, fragments were separated by electrophoresis using 3% of agarose gel. Fragments of DNA stained with ethidium bromide were observed under UV light using a transilluminator and recorded by digital photos. The molecular weight marker 1 kb Plus DNA Ladder (Invitrogen®) was utilized.

#### 2.4. Sequencing

To obtain the sequences, the ET Dye Terminator (Amersham Biosciences) sequencing kit, compatible with the MEGA BACE 1000 platform, was used, following the guidelines provided by the manufacturer.

The sequences obtained were submitted to BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1997) in order to identify their similarity with sequences of the GenBank. Sequence alignment was performed using CLUSTAL W (Higgins et al., 1994) with manual adjustments. For phylogenetic analysis, the MEGA 3.1 program (Kumar et al., 2004) was used. The phylogenetic analyses were performed by Neighbor Joining with Kimura 2 Parameter and Maximum Parsimony and Maximum Likelihood utilizing the PHYLIP package (Felsenstein, 1993). The consensus tree was obtained after bootstrap analysis, with 1000 replications. For the construction of the phylogenetic tree was used *Eimeria tenella* (AF026388) as the outgroup.

For comparative phylogenetic analysis, the following sequences were retrieved from the GenBank: *C. meleagridis* (AF112574, AF329187 and AF404821); *C. parvum* ferret genotype (AF112572); *C. parvum* mouse genotype (AF112571); *C. suis* (AF115377 and AF108861); *C. wrairi* (AY115378); *C. parvum* (DQ067566, AF164102 and AF093490); *C. hominis* (DQ286403, AF093489, AF108865 and AJ849464); *C. parvum* rabbit genotype (AY112573); *C. saurophyllum* (AF112573); *C. felis* (AF159113, AF108862 and AF112575); *Cryptosporidium* sp. isolate N (AF262332); *C. canis* (AY120909, AJ493209, AF112576 and AB210854); *Cryptosporidium* sp. Deer genotype (AY120910); *C. bovis* (AY120911 and AY731305); *C. baileyi* (AF093495, AY954884 and AF262324); *C. serpentis* (AF093502); *C. galli*: (AY168847); *C. muris* (AF093498) and *C. andersoni* (AB089285).

### 3. Results

#### 3.1. Public market

In the first fecal samplings and analysis at the public market, *Cryptosporidium* oocysts were only found in the cages holding chickens, ducks, quails and guinea pigs. Subsequently four ducks, three chickens, three quails and two guinea pigs were acquired and kept in the laboratory for further fecal sampling. All these animals showed only *Cryptosporidium* sp. oocysts and no other parasites were detected during microscopic analysis of slide preparations.

For DNA extraction, one fecal sample from each duck, one from a quail, one from a chicken and one from each guinea pig were selected. The RFLP of 18S rDNA amplified products digested with *SspI* showed that *Cryptosporidium* sp. from duck and quail samples had a restriction pattern similar to that described for *C. baileyi* (Fig. 1A). The *Cryptosporidium* sample from the chicken showed a restriction pattern compatible with *C. meleagridis*, when digested with *SspI* (Fig. 1A) and *VspI* (fragments: 468, 186 and 168 bp) (Figure not shown).

The samples from guinea pigs did not digest by *SspI* (Fig. 1A) but showed fragments of 689 and 110 bp when treated with *VspI* (Figure not shown)

All digestion patterns obtained in this study were compared to those established by Xiao et al. (2004).

For species confirmation and phylogenetic analysis, each PCR product was sequenced. The sequences obtained were deposited with the GenBank and received the following accession numbers: *C. baileyi* (DQ885335, DQ885339 and DQ885340); *C. meleagridis* (DQ885341).

The sequences of *Cryptosporidium* sp. isolates from guinea pigs (accession numbers DQ885337 and DQ885338) showed no similarity to any known *Cryptosporidium* sp. at the specific level.

#### 3.2. Cat shelter

A total of 30 fecal samples from cats were screened for *Cryptosporidium* and other parasites. All of them showed the presence of helminth eggs or protozoan oocysts and cysts. Multiple parasitism was observed in 90% of cat fecal samples, varying from 2 to more than 5 other species. The distribution of parasites species in the total samples were as follows: *Toxocara* sp. was found in 26 samples (86.7%), *Ancylostoma* sp. in 14 samples (46.7%), *Platynosomum* in 6 samples (20%), *Dipylidium caninum* in 4 samples (13.3%), *Cystoisospora* sp.

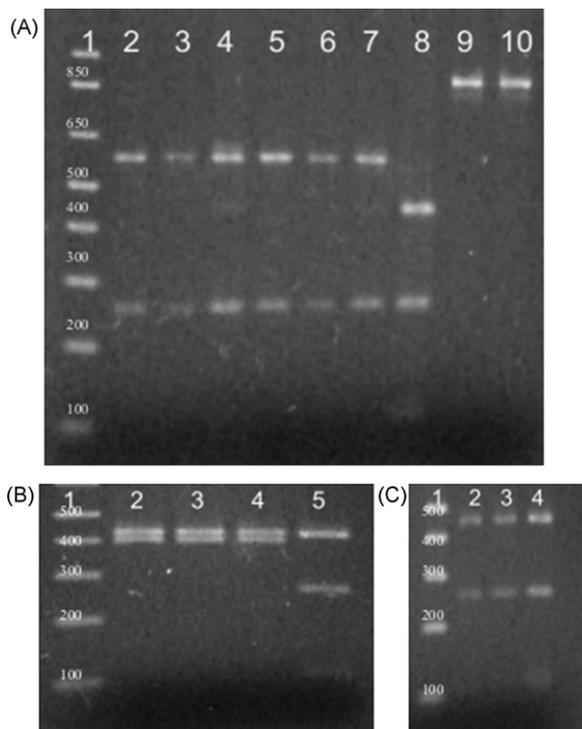


Fig. 1. PCR-RFLP of the *SspI* digestion from *Cryptosporidium* 18S rDNA from domestic animals. (A) Lane 1: molecular weight marker, lanes 2–6: *C. baileyi* from domestic ducks (fragment size: 253 and 578 bp), lane 7: *C. baileyi* from a quail (fragment size: 253 and 578 bp), lane 8: *C. meleagridis* from a chicken (fragment size: 117, 264 and 442 bp), lanes 9 and 10 not digested *Cryptosporidium* sp. 18S rDNA fragment from guinea pigs (860 bp). Variation of molecular weights  $\pm 3\%$ . (B) Lane 1: molecular weight marker, lanes 2–4: *C. felis* from cats (fragment size: 447 and 418 bp), lane 5: *C. canis* (fragment size: 437, 268 and 110 bp). Variation of molecular weights  $\pm 3\%$ . (C) Lane 1: molecular weight marker, lanes 2–4: *C. parvum* (fragment size: 461, 260 and 110 bp). Variation of molecular weights  $\pm 3\%$ .

in 4 samples (13.3%), *Sarcocystis* sp. in a single sample (3.3%) and *Giardia* sp. cysts in 18 samples (60.0%).

During the analysis of parasitism in the four dog fecal samples, we observed that all of them contained *Giardia* sp. cysts, *Toxocara* sp. eggs and *Ancylostoma* sp. eggs. However, only one (25%) had *Cryptosporidium* sp., and *Trichuris* sp. eggs were found in only one other sample.

Nine out of the 30 cat samples had *Cryptosporidium* sp. oocysts (30%), but only six displayed more than four oocysts per slide under microscopic examination. The six cats and the single dog fecal sample infected by *Cryptosporidium* were further investigated with molecular biology techniques to identify the species involved.

The RFLP observed after digestion of PCR products from the dog with *SspI* (Fig. 1-A) and *VspI* (fragment

size: 705 and 110 bp. Figure not shown) was found to be compatible with *C. canis*, as reported by Xiao et al. (2004). The RFLP derived from three cat samples were similar and compatible with those previously described for *C. felis* (Fig. 1B).

The other three cat samples did not amplify, even after several attempts, probably due to the presence of inhibitor contaminants in the fecal samples.

### 3.3. Dairy calves

Ten out of the 56 fecal samples from dairy calves contained *Cryptosporidium* oocysts. Three were selected for PCR and RFLP analysis due to the presence of a high number of oocysts. The RFLP pattern using *SspI* (Fig. 1C) and *VspI* (Fragment size: 689 and 112 bp. Figure not shown), showed fragments compatible with those previously observed by Xiao et al. (2004) for *C. parvum*.

One of these samples was sequenced and deposited with the GenBank, receiving accession number DQ885333.

### 3.4. Phylogeny

The species *C. parvum*, *C. baileyi* and *C. meleagridis* showed 99% similarity when compared to respective sequences available in the GenBank. The sequences of *C. felis* and *C. canis* obtained in the present study showed respective similarities of 98% and 97% when compared to sequences in the GenBank. Table 1 shows the genetic distances between several sequences of *C. felis* and *C. canis* derived from this study or retrieved from the GenBank. It is interesting to note that the Brazilian sequences show a greater genetic distance than that between sequences from of *C. felis* and *C. canis* available in the GenBank.

Table 2 shows the genetic distances between the *Cryptosporidium* sp. from Guinea pigs and known species of *Cryptosporidium* from the GenBank, including one unknown species from surface water (Xiao et al., 2000a).

In the phylogenetic analysis the isolates obtained here grouped with already described species, maintaining relative host specificity (Fig. 2). The topology of the phylogenetic consensus tree obtained by Neighbor Joining was confirmed by Maximum Parsimony analysis and Maximum Likelihood. It was not possible to identify the sequences of *Cryptosporidium* obtained from Guinea pigs, because the two sequences clustered together and out of the clusters of previously known species. The *Cryptosporidium* isolates from guinea pigs

Table 1

Genetic distances using Kimura-2 Parameter, comparing the Brazilian sequence of *Cryptosporidium canis* and *C. felis* and sequences retrieved from GenBank

	<i>C. canis</i> (this work)	<i>C. canis</i> AF112576	<i>C. canis</i> AB210854	<i>C. canis</i> AY120909	<i>C. canis</i> AJ493209	<i>C. felis</i> (this work)	<i>C. felis</i> AF112575	<i>C. felis</i> AF159113	<i>C. felis</i> AF108862
<i>C. canis</i> (this work)									
<i>C. canis</i> AF112576	<b>0.008</b>								
<i>C. canis</i> AB210854	<b>0.008</b>	0.000							
<i>C. canis</i> AY120909	<b>0.010</b>	0.002	0.002						
<i>C. canis</i> AJ493209	<b>0.008</b>	0.000	0.000	0.002					
<i>C. felis</i> (this work)	0.072	0.064	0.064	0.062	0.064				
<i>C. felis</i> AF112575	0.066	0.058	0.058	0.055	0.058	<b>0.006</b>			
<i>C. felis</i> AF159113	0.068	0.060	0.060	0.058	0.060	<b>0.008</b>	0.002		
<i>C. felis</i> AF108862	0.066	0.058	0.058	0.055	0.058	<b>0.006</b>	0.000	0.002	
<i>Eimeria tenella</i> AF026388	0.273	0.267	0.267	0.265	0.267	0.269	0.261	0.263	0.261

grouped together with intestinal *Cryptosporidium* species, being genetically related to *C. felis*.

#### 4. Discussion

In the present study, four species of *Cryptosporidium* with proven zoonotic potential were diagnosed with molecular tools: *C. parvum*, *C. meleagridis*, *C. felis* and *C. canis*. In the dogs and cats from the animal shelter, the presence of other parasites resulting in double or multiple infections with helminths and other protozoans shows the precarious sanitary condition of these animals. As *Cryptosporidium* is an important opportunistic parasite, this intense parasitism could have collaborated to debilitate the hosts, favoring the infection of cats and the dog with *C. felis* and *C. canis*, respectively.

In a previous study, the occurrence of *Cryptosporidium* sp. in 25% of 20 cats maintained in a shelter was reported (Huber et al., 2002). However, in general the occurrences of infection by *Cryptosporidium* sp. in cats mentioned in other studies are less than 12.1% (Mtambo et al., 1991; Hill et al., 2000; Spain et al., 2001). It is noteworthy that despite the high environmental contamination with *C. felis*, the dog was infected with *C. canis*, indicating a certain degree of host specificity. Abe et al. (2002) also diagnosed only *C. canis* naturally infecting 140 fecal samples of dogs. Besides this, to date there is no reported case in the consulted literature mentioning species of *Cryptosporidium* other than *C. felis* naturally infecting cats. The same occurs for the natural infection of dogs with *C. canis*, although genotyping studies of natural *Cryptosporidium* infections in dogs and cats are still rare.

According to Scorza et al. (2003), the different distribution of oocysts in fecal samples can result in insufficient oocysts in the aliquot samples used in

diagnostic procedures. This fact, associated with the failure of DNA release during extraction, the absorption of the DNA by fecal debris and the presence of inhibitors, can make the use of the PCR in fecal samples quite difficult. These factors may have been responsible for the lack of amplification of DNA from three fecal samples of cats that were positive for *Cryptosporidium* during microscopic examination.

The sequence analysis and phylogenetic grouping suggests that the *Cryptosporidium* infecting guinea pigs represents a genotype still not described. This finding calls attention to the fact that even in well-known host species, as the guinea pig, it may be possible to find new parasite genotypes. However, rather detailed studies need to be performed to elucidate the phylogenetic relationship of this isolate and other *Cryptosporidium* sp.

A greater genetic distance was found between the *C. felis* and *C. canis* isolated in Brazil when compared with the sequences retrieved from the GenBank. Some theories explaining *Cryptosporidium* genotype formation and speciation exist to-date. Host-adaptation seems to be important for the speciation in this genus as supported by phylogenetic analyses, where genetically related hosts often are parasitized with related *Cryptosporidium* species (Xiao et al., 2002). This presumes the co-evolution of host and parasites for a long time, since the separation of gastric and intestinal *Cryptosporidium* species seems to have occurred before the emergence of reptiles (Xiao et al., 2002).

However this may be only one factor that can lead to speciation. Host-adaptation may also occur, when one species adapts to a new host. This is presumed to have occurred with *C. meleagridis*, adapting from birds to mammals, and *C. parvum*, adapting from rodents to ruminants (Xiao et al., 2002, 2004) and possibly in other species, too. It is widely reported that different species

Table 2  
Genetic distances using Kimura-2 Parameter, comparing the Brazilian sequences of *Cryptosporidium* from Guinea pigs and sequences retrieved from GenBank

	<i>Cryptosporidium</i> from Guinea pig O92	<i>Cryptosporidium</i> from Guinea pig O93	<i>C. felis</i> AF112575	<i>C. sp. N</i> AF262332	<i>C. wrairi</i> AY115378	RABBIT GENOT AY120901	<i>C. parvum</i> AF164102	MOUSE GENOT AF112571	FERRET AF112572	<i>C. canis</i> AB210854	<i>C. baileyi</i> AF262324	DEER GENOT AY120910	<i>C. muris</i> AF093498
<i>Cryptosporidium</i> from Guinea pig O93	0.003												
<i>C. felis</i> AF112575	0.037	0.040											
<i>C.sp. N</i> AF262332	0.045	0.048	0.050										
<i>C.wrairi</i> AY115378	0.048	0.050	0.037	0.037									
RABBIT GENOT AY120901	0.048	0.050	0.039	0.034	0.013								
<i>C. parvum</i> AF164102	0.050	0.053	0.039	0.040	0.003	0.010							
MOUSE GENOT AF112571	0.053	0.056	0.039	0.042	0.008	0.010	0.005						
FERRET AF112572	0.053	0.056	0.039	0.042	0.008	0.010	0.005	0.005					
<i>C. canis</i> AB210854	0.064	0.067	0.042	0.048	0.031	0.039	0.034	0.039	0.039				
<i>C. baileyi</i> AF262324	0.076	0.078	0.059	0.059	0.056	0.056	0.056	0.062	0.062	0.053			
DEER GENOT AY120910	0.076	0.078	0.059	0.059	0.053	0.048	0.053	0.053	0.053	0.050	0.045		
<i>C. muris</i> AF093498	0.113	0.116	0.102	0.113	0.096	0.104	0.099	0.099	0.104	0.099	0.078	0.107	
<i>E. tenella</i> AF026388	0.263	0.267	0.266	0.255	0.282	0.282	0.286	0.285	0.289	0.286	0.285	0.292	0.262

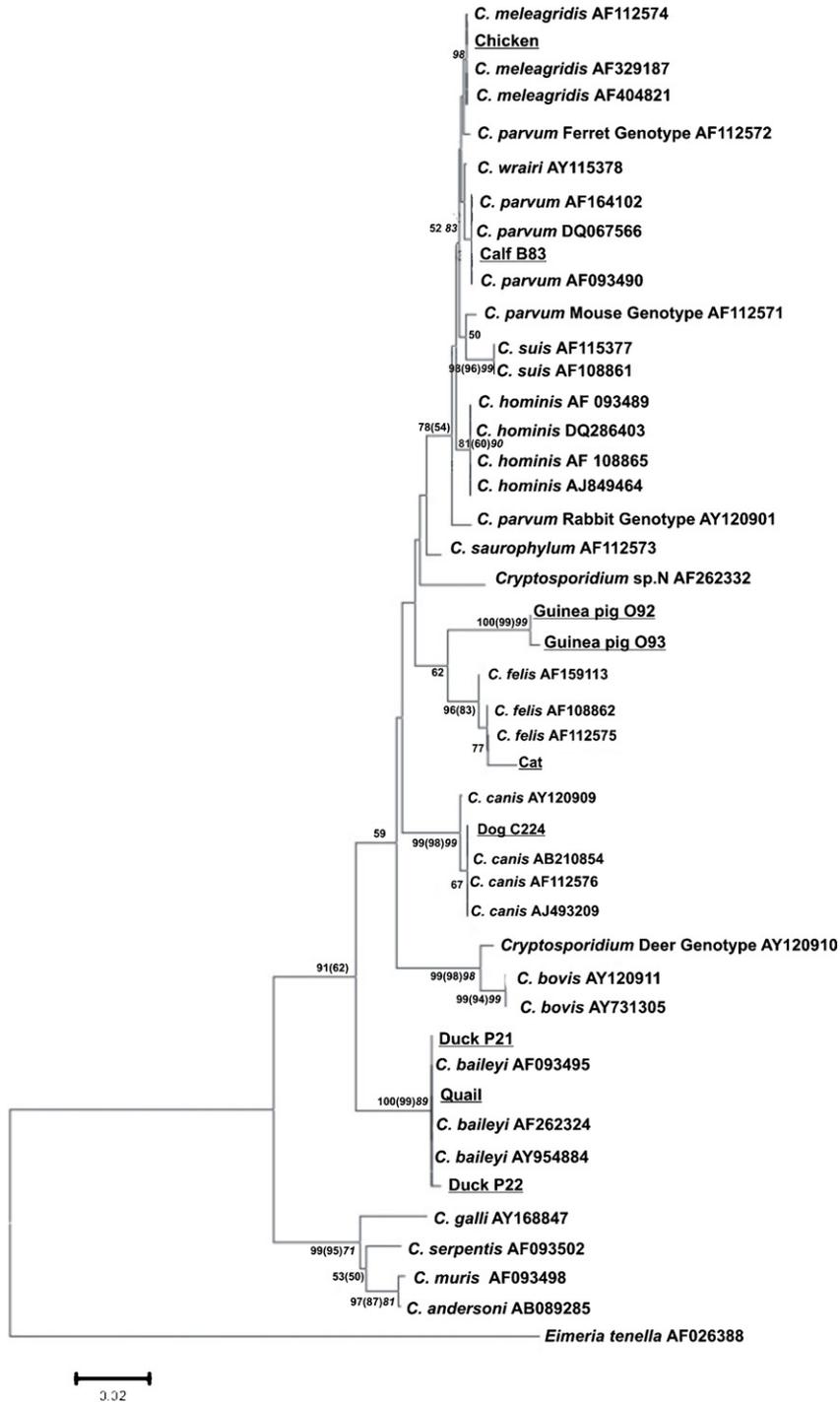


Fig. 2. Phylogenetic relationship among *Cryptosporidium* sequences retrieved from GenBank and sequences from Brazil (underlined) inferred by a neighbor-joining analysis of the partial 18S rDNA and confirmed by maximum parsimony and maximum likelihood. Values on branches are percent bootstrapping using 1000 replicates. Only values >50% are shown. Those for neighbor-joining are in normal style, maximum parsimony in parenthesis and maximum likelihood in italic. The Brazilian isolates grouped with already described species, except for the isolates from guinea pigs that clustered together, sharing a branch with *C. felis*.

of *Cryptosporidium* can parasitize the same host, as occurs with *C. parvum*, *C. hominis* and *C. meleagridis* (Cama et al., 2003; Pieniazek et al., 1999; Xiao et al., 2001). Genetic recombination, especially in mixed infections, may be responsible for emergence of new genotypes, as reported for *Cryptosporidium parvum* and mixed infection of *C. parvum* and *C. hominis* (Feng et al., 2002; Tanriverdi and Widmer, 2006). In other cases the fast reproduction of the genera and the ability to establish a clonal population may lead to the emergence of distinct genetic populations inside a group of hosts in a relative short time, as described for *C. parvum* (Tanriverdi et al., 2006). According to Tanriverdi et al. (2006), this can explain the frequent detection of genotypes specific to hosts whose taxonomic status is unknown, as occurs in studies of environmental oocyst isolates from surface water and geographically restricted genotypes of *C. hominis*.

These facts could explain the greater genetic distances observed between the genetic sequences of *C. canis* and *C. felis* from this study when compared to reference sequences from GenBank as well as the *Cryptosporidium* sp. genotype from guinea pigs. However, for a more comprehensive description of *Cryptosporidium* species in Brazil, extensive studies are extremely important, including biological aspects associated with molecular techniques.

Despite the existence of epidemiological surveys on animal cryptosporidiosis in Brazil, there are only a few studies using molecular techniques. Therefore, the results presented in this study are pioneering in this area with respect to *Cryptosporidium* sp. in Brazil.

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