

Occurrence and molecular characterization of *Cryptosporidium* spp. isolated from domestic animals in a rural area surrounding Atlantic dry forest fragments in Teodoro Sampaio municipality, State of São Paulo, Brazil

Ocorrência e caracterização molecular de *Cryptosporidium* spp. isolados de animais domésticos de propriedades rurais circunvizinhas a fragmentos de Floresta Atlântica Seca do Estado de São Paulo, Brasil

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Received August 31, 2010

Accepted September 24, 2010

Abstract

The aim of this study was to assess the occurrence of *Cryptosporidium* in domestic animals in rural properties surrounding rain forest fragments within the municipality of Teodoro Sampaio, southeastern Brazil. Conventional sucrose flotation method followed by molecular characterization of the parasites by sequencing PCR products amplified from SSU rRNA gene were used. Stool samples were collected from domestic animals raised as pets and livestock in all rural properties surrounding three forest fragments. Samples from cattle (197), equine (63), pigs (25), sheep (11), and dogs (28) were collected from 98 rural properties. The frequency of occurrence of *Cryptosporidium* within each animal species was 3.0% (6/197) among cattle and 10.7% (3/28) among dogs. *Cryptosporidium* was not detected in stool samples from equine, sheep, and pigs. All sequences obtained from the six samples of calves showed molecular identity with *Cryptosporidium andersoni* while all sequences from dog samples were similar to *C. canis*. The frequency of occurrence of *Cryptosporidium* in these domestic animal species was low. The absence of *C. parvum* in the present study suggests that the zoonotic cycle of cryptosporidiosis may not be relevant in the region studied. The presence of *Cryptosporidium* species seldom described in humans may be, otherwise, important for the wild fauna as these animals are a source of infection and dissemination of this protozoan to other animal species. The impact and magnitude of infection by *C. andersoni* in wild ruminants and *C. canis* in wild canids have to be assessed in future studies to better understand the actual importance of these species in this region.

Keywords: Cryptosporidiosis, molecular characterization, SSU rRNA, Atlantic Dry Forest, domestic animals.

Resumo

O objetivo deste estudo foi avaliar a ocorrência de *Cryptosporidium*, em animais domésticos de propriedades rurais ao redor de fragmentos de mata Atlântica de interior no município de Teodoro Sampaio, por exame convencional de flutuação em solução de sacarose, seguido de caracterização molecular dos parasitas através do sequenciamento dos produtos amplificados na PCR do gene SSU rRNA. Foram coletadas amostras fecais de animais domésticos criados para subsistência e estimação nas propriedades rurais do entorno de três fragmentos florestais. Amostras de bovinos (197), equinos (63), suínos (25), ovinos (11) e cães (28) foram coletadas de 98 propriedades rurais. A ocorrência de *Cryptosporidium* para a espécie bovina foi de 3,0% (6/197); para os cães, de 10,7% (3/28); e para os demais animais os resultados foram negativos. Todas as sequências obtidas das seis amostras de bovinos apresentaram identidade molecular com *Cryptosporidium andersoni*, enquanto as sequências oriundas de amostras de fezes de cães revelaram-se similares

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ao *C. canis*. A ocorrência do *Cryptosporidium* entre os animais estudados foi baixa. Diante dos resultados do presente estudo, o ciclo zoonótico da criptosporidiose parece ter menos importância nesta região. A presença de espécies de *Cryptosporidium* ainda pouco relatadas em humanos pode ser, por outro lado, importante para a fauna silvestre, uma vez que estes animais podem ser considerados como uma fonte de infecção e disseminação deste protozoário. O impacto e a magnitude da infecção de *C. andersoni*, em ruminantes selvagens, e de *C. canis*, em cães silvestres, deve ser avaliado em estudos futuros, com intuito de verificar a real importância dessas espécies nesta região.

Palavras-chave: Criptosporidiose, caracterização molecular, SSU rRNA, Mata Atlântica de interior, animais domésticos.

Introduction

Pontal do Paranapanema is a region in the westernmost of São Paulo State (INSTITUTO FLORESTAL, 2009) that contains a number of rain forest fragments. This region contains the largest preserved area of Atlantic Forest that still exist inland São Paulo State (Atlantic dry forest). The largest Atlantic dry forest fragment in this region is the Morro do Diabo State Park (MDSP), a vast area of 33,845.33 ha within Pontal do Paranapanema region in the Teodoro Sampaio municipality. Around MDSP, there are 63 small properties of agrarian reform settlements and 10 large farms with monoculture of sugar cane and livestock. The small properties are owned by low income farmers which live from subsistence agriculture.

Endoparasites, like worms and protozoans, are of great impact on public and animal health around the world, mainly in developing countries. Cryptosporidiosis is an endoparasitosis caused by coccidians of *Cryptosporidium* genus which can infect a wide range of hosts, e.g., reptiles, birds and mammals, including humans (SMITH et al., 2007). The occurrence of *Cryptosporidium* in animals is a public health problem due to the zoonotic potential of some species of this genus. In addition, due to low host specificity of *Cryptosporidium*, the domestic fauna may pose the sylvatic fauna at risk of infection, as domestic animals may act as source of infection for wild animals (XIAO et al., 2000).

The small subunit rRNA-based genotyping is widely used to compare the differences in genetic diversity among *Cryptosporidium* species. By using this marker, it is possible to differentiate all the 20 recognized species and more than 40 genotypes of *Cryptosporidium* (SMITH et al., 2007; XIAO; FAYER, 2008). DNA sequences of other markers, such as the *Cryptosporidium* oocyst wall protein (COWP) gene and the 70 kDa heat shock protein (HSP70) gene have also been used (FAYER et al., 2001; MORGAN-RYAN et al., 2002).

The aim of this study was to assess the occurrence of *Cryptosporidium* in rural properties surrounding three rain forest fragments within the Teodoro Sampaio municipality. Coprological methods and further molecular characterization of the parasites by sequencing fragments of PCR products amplified from small subunit (SSU) rRNA gene were used.

Material and Methods

In June 2007, stool samples were collected from domestic animals raised as pets and for subsistence at all rural properties surrounding MDSP and from two smaller forest fragments

(Figure 1). A total of 98 rural properties were sampled. All the animals sampled were mixed breed.

Cattle samples were collected from 79 properties, equine from 46, pigs from 20, sheep from nine, and dogs from 21. Only fresh samples were collected directly from the ground, fixed in 2.0% potassium dichromate and stored at 5.0 °C for up to three weeks. The samples were collected with the help of the owners, providing that same animal was not sampled twice. They were sent to the laboratory immediately after collection in plastic bags under refrigeration.

Altogether, samples were collected from 197 bovine (118 less than 3 months old), 63 equine (5 less than 3 months old), 25 pigs (2 less than 3 months old), 11 sheep (1 less than 3 months old), and 28 dogs (1 less than 3 months old).

Stool samples were examined for oocysts of *Cryptosporidium* spp. by a conventional sucrose flotation method. Floated material was transferred to a slide and examined by light microscopy. When 4-8 µm sized oocysts were observed, the slide was washed and DNA was extracted from the material as previously described (THOMAZ et al., 2007).

To amplify fragments of the SSU rRNA gene, a nested-PCR was used (THOMAZ et al., 2007). The nested-PCR products were sequenced using secondary PCR primers and Big Dye chemistry (Applied Biosystems, Foster City, California). Sequencing products were analyzed on an ABI377 automated sequencer. Both strands of each nested-PCR products were sequenced at least twice to increase the confidence of sequencing. The sequences were assembled and the contig formed with the phred-base-calling and the phrap-assembly tool available in the suite Codoncode aligner v.1.5.2. (Codoncode Corp. Dedham, MA, USA). SSU rRNA sequences of *Cryptosporidium* spp. were aligned and the resulting alignment matrix was used as input to Molecular Evolutionary Genetics Analysis (MEGA) software version 4 (TAMURA et al., 2007). Phylogeny was reconstructed with distance matrix method using the maximum composite likelihood.

Results

The occurrence of *Cryptosporidium* was detected in eight of the 98 properties (8.2%). In six of them, the parasites were found only in feces of young calves less than 3 months old and in two properties the parasites were found only in feces from dogs. In one of the properties an adult dog was eliminating the parasite while in the other property the parasite was found in stool samples from a puppy and an adult dog.

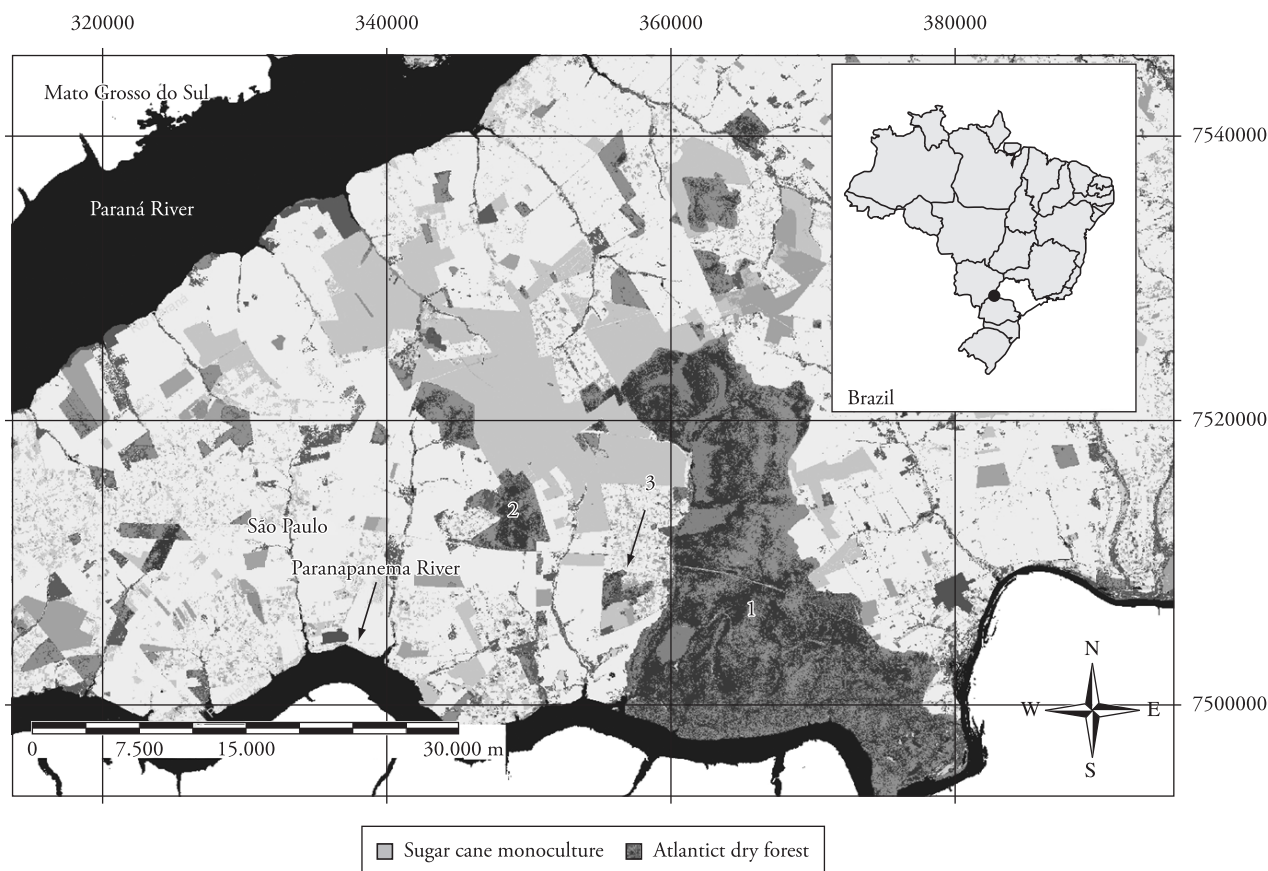


Figure 1. Pontal do Paranapanema, SP, Brazil, with fragments of dry forests. The dark area (spot) in the upper right was magnified in the larger map: 1) Morro do Diabo State Park located at 22° 24' to 22° 38' S and 52° 09' to 52° 23' W. 2) Ribeirão Bonito forest fragment is located at 22° 30' to 22° 32' S and 52° 23' to 52° 24' W. 3) Tucano forest fragment is located at 22° 27' to 22° 29' S and 52° 27' to 52° 29' W.

The frequency of occurrence of *Cryptosporidium* within each animal species was 3.0% (6/197) among cattle and 10.7% (3/28) among dogs. *Cryptosporidium* was not detected in stool samples from equine, ovine, and pigs.

All the sequences from calves showed molecular identity with *C. andersoni* while all sequences from dogs were revealed a similarity to *C. canis* (Figure 2). The SSU rRNA nucleotide sequences of five isolates of cattle revealed 100% identity with homologous sequences of *C. andersoni* (identical to sequence AF093496). The nucleotide sequence of the remaining isolates of cattle was also 100% similar to AF093496 except for a thymidine insertion. The SSU rRNA nucleotide sequences of all the isolates of dogs revealed 100% identity with homologous sequences of *C. canis* (identical to sequence AB210854). The sequences were deposited in Genbank under the accession numbers GU365874 – GU365876.

Discussion

The frequency of occurrence of *Cryptosporidium* among domestic animals raised in rural properties surrounding the forests in Pontal do Paranapanema was low. In ruminants, cattle in particular, the occurrences of the low specific *C. parvum*, and the host-adapted species *C. andersoni* and *C. bovis* have been reported.

Cryptosporidium andersoni is considered of low pathogenicity (ANDERSON, 1991) and rarely pose risk of infection to humans (LEONI et al., 2006). In dogs, two species of *Cryptosporidium* can be found, *C. parvum* and *C. canis*. (FAYER et al., 2001) Within the genus *Cryptosporidium*, five species, *C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis* and *C. canis* may infect immunocompetent and immunocompromised humans. Among them, the latter one is the rarest in humans (XIAO; FENG, 2008). The results of the present study showed that the zoonotic cycle of cryptosporidiosis appears to be of less relevance in the region studied.

The frequency of bovines positive for the presence of *Cryptosporidium* was equivalent to 3.0%, a low frequency compared to those reported in other studies. Thomaz et al. (2007) found 17.1% (21/123) of positive samples of *Cryptosporidium* spp. in calves from dairy farms in the State of São Paulo (THOMAZ et al., 2007). Huber et al. (2005) found 10 positive samples (5.6%) in dairy cattle in the State of Rio de Janeiro, while Langoni et al. (2004) found 21.2% of *Cryptosporidium* positivity in 203 fecal samples of calves from several farms in the State of São Paulo. The exclusive occurrence of *C. andersoni* in bovines in this study is a result which is markedly different from those reported elsewhere in which *C. parvum* predominates. Thomaz et al. (2007) found 88.9% of *C. parvum* and 11.1% of *C. bovis* in cattle, whereas Huber et al. (2005) found *C. parvum* only. In other regions of the

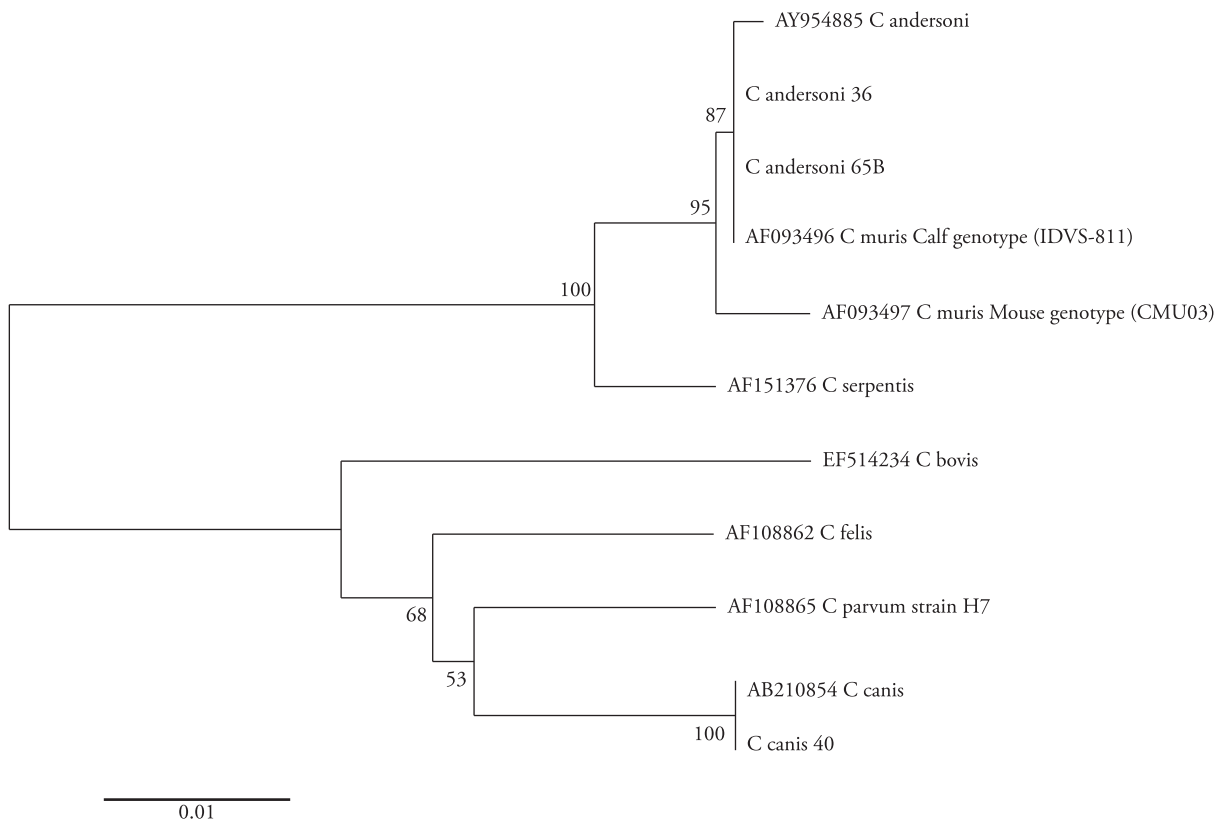


Figure 2. Phylogenetic relationships among *Cryptosporidium* spp. isolates inferred from the alignment of the SSU-rRNA sequences and distance analysis. The tree is unrooted. The numbers indicate the number of times that the branches are supported after 1,000 bootstrap replicates. Taxons identified in this study: *C. andersoni* 36, *C. andersoni* 65B and *C. canis* 40.

world *C. parvum* and *C. andersoni* were the two most common genotypes in bovines (XIAO; HERD, 1994; WADE et al., 2000; HUETINK et al., 2001; ENEMARK et al., 2002; PENG et al., 2003). In Maryland, United States, *C. parvum* accounted for 97% of infections in pre-weaned calves but only 4 and 0% of infections in post-weaned calves and heifers, respectively (SANTÍN et al., 2008).

Cryptosporidium infection in pigs, sheep and goats can be zoonotic as these hosts are susceptible to *C. parvum* infection. The fact that *C. parvum* was not found in these animals may indicate that the role of this species in the epidemiological cycle of cryptosporidiosis is of less zoonotic importance in the region studied. *Cryptosporidium* is recognized as one of the main causes of diarrhea in lambs (de GRAAF et al., 1999), causing high morbidity and mortality and major economic losses (CASEMORE et al., 1997). In pigs and equines *Cryptosporidium* appear to be of less zoonotic importance, although diarrhea in post-weaning piglets and reduction in growth rate, food absorption and weight loss in equine have already been described (MISIC et al., 2003; GRINBERG et al., 2003).

The proportion of positive samples among dogs was 10.7% (3/28 animals), a frequency similar to that reported by Thomaz et al. (2007) who found 12.5% (15/120) of positivity in dogs from an urban area and also found *C. canis* only. Other studies in urban

areas in Brazil also revealed low frequency of *Cryptosporidium* in dogs. Gennari et al. (1999) have found 2.8% of 353 animals shedding *Cryptosporidium*. Funada et al. (2007) found 2.4% of positivity for *Cryptosporidium* in 1,755 samples of dogs, collected between January 2000 and December 2004. In Londrina, Paraná State, Brazil, 2.2% of samples of dogs were positive to *Cryptosporidium* sp. (NAVARRO et al., 1997) and in the city of Rio de Janeiro, Rio de Janeiro State, Brazil, the positivity in dogs was 2.4% (HUBER et al., 2005). However, it is worth mentioning that none of the aforementioned studies included molecular identification of the parasite.

Although the results of our study potentially indicate a low frequency of occurrence of *Cryptosporidium* in domestic animals, three points should be highlighted. First, the samples from animals other than bovines were predominantly from adults, which can explain the low frequency of *Cryptosporidium*. It is well known that oocyst shedding is more common in younger animals (THOMPSON et al., 2005). Second, sample collection was performed during the dry season (from April to September) which may lead to an underestimation of the frequency of infection because of lower transmission of the parasites during this period. It is noteworthy that oocyst dispersion and transmission are greatly favored in wet environment during the rainy season. Third, the presence of *Cryptosporidium* of less relevance in terms of zoonotic

transmission may be, otherwise, important for the wild fauna as these animals can be considered a source of *Cryptosporidium* infection. The impact and magnitude of infection by *C. andersoni* in wild ruminants and by *C. canis* in wild canids need to be assessed in future studies to better understand the actual importance of this genus of parasite for the wild fauna living in the MDSP and other rain forest fragments.

Acknowledgments

The authors thank Alexandre Uezo and the Institute of Ecologic Research. L. J. Richtzenhain and R. M. Soares are recipients of a productivity fellowship from the National Council for Scientific and Technological Development (CNPq).

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