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Molecular characterisation of *Cryptosporidium* spp. in lambs in the South Central region of the State of São Paulo

[*Caracterização molecular de* Cryptosporidium *spp. em cordeiros na região centro sul do Estado de São Paulo*]

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ABSTRACT

Considering the proximity of sheep farmers to animals that are possibly diseased or releasing fecal oocysts into the environment and the marked pathogenicity in lambs, the aim of this study was to determine the occurrence and to molecularly characterize the infection by *Cryptosporidium* spp. in lambs in the South Central region of the state of São Paulo, Brazil. A total of 193 fecal samples were collected from sheep of several breeds, males and females, aged up to one year. Polymerase chain reaction (*nested*-PCR) was used to amplify DNA fragments from the subunit 18S rRNA gene and indicated 15% positivity; sequencing of amplified fragments was possible for 19 samples. Analysis of the obtained sequences showed that the identified species were *Cryptosporidium xiaoi* for 15 samples, constituting thus the first molecular characterization study of this *Cryptosporidium* species in Brazil. *Cryptosporidium ubiquitum* was identified for three samples and *Cryptosporidium meleagridis* for one sample; the latter two are considered zoonotic species.

Keywords: cryptosporidiosis, sheep, *nested*-PCR, genotype

RESUMO

Devido à proximidade de criadores de ovinos com animais possivelmente doentes e/ou eliminando oocistos fecais no ambiente e pela acentuada patogenicidade em cordeiros o objetivo foi, determinar a ocorrência e caracterizar molecularmente a infecção por Cryptosporidium *spp. em cordeiros na região Centro Sul do Estado de São Paulo, Brasil. Num total de 193 amostras de fezes foram coletadas de ovinos de diversas raças, machos e fêmeas, com idade de até um ano. Por meio da reação em cadeia da polimerase* (nested *PCR) para a amplificação de fragmentos de DNA a partir do gene da subunidade 18S do rRNA houve positividade de 15% e o sequenciamento dos fragmentos amplificados foi possível em 19 amostras. A análise das sequências obtidas mostraram que as espécies identificadas nesses animais foram* Cryptosporidium xiaoi *em 15 amostras, sendo o primeiro estudo de caracterização molecular desta espécie de* Cryptosporidium *no Brasil.* Cryptosporidium ubiquitum *em três amostras, e* Cryptosporidium meleagridis *em uma amostra, sendo estas duas últimas consideradas espécies zoonóticas.*

Palavras-chave: cryptosporidiose, ovinos, *nested*-PCR, genótipo

INTRODUCTION

Cryptosporidiosis interferes in the life quality of men and arouses great public health interest due to its high occurrence (Carvalho, 2009). This is in part a consequence of the increased number of bearers of Acquired Immunodeficiency Syndrome (AIDS) associated with opportunistic infection and patients undergoing immunosuppression therapy (Fayer, 2010).

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The protozoan *Cryptosporidium* is potentially zoonotic. In the last years, the interest in the study of this genus has grown, especially when molecular techniques are used and several species, genotypes and subtypes of this parasite are described (Plutzer and Karanis, 2009). Currently, there are more than 22 species, of which 12 are in mammals, and over 61 genotypes described for *Cryptosporidium*, determined according to the host and genetic analyses (Xiao, 2010).

Molecular characterization of isolates of different origins (human, animal and environment) has been widely used to investigate the zoonotic potential of species or genotypes of this protozoan (Xiao and Fayer, 2008). Since this parasite is capable of infecting several hosts and is constantly present in the environment, humans can acquire the infection by direct contact with other infected people (anthroponotic) or animals (zoonotic) and by ingestion of contaminated food or water (Xiao, 2010).

By means of molecular techniques, species of *Cryptosporidium* have been observed in fecal samples from lambs in countries like the United States (Fayer and Santín, 2009), United Kingdom (Mueller-Doblies *et al*., 2008), Italy (Paoletti *et al*., 2009), Spain (Quílez *et al*., 2008), Tunisia (Soltane *et al*., 2007), China (Wang *et al*., 2010), Australia (Yang *et al*., 2009) and Brazil (Féres *et al*., 2009), and the main species responsible for infections in sheep are: *C. parvum, C. xiaoi* and *C. ubiquitum* (Fayer and Santín, 2009; Fayer *et al*., 2010).

Considering the proximity of sheep farmers to animals that are possibly diseased and/or releasing fecal oocysts into the environment and the marked pathogenicity in lambs, the present study aimed to determine the occurrence of infection by *Cryptosporidium* spp. and molecularly identify the involved species in fecal samples from lambs in the South Central region of the state of São Paulo.

MATERIAL AND METHODS

In July 2011, fecal samples were collected from 193 lambs aged up to one year, of which 38 were male and 155 female, from the Center-South region of São Paulo State, Brazil. The system employed in all three sheep farms was semi-intensive, and the herd composition was varied and composed of crossbreds (42) and specimens of the breeds were Texel (129) and Santa Inês (22). The animals were weaned at 60 and 90 days of age.

Fecal samples were obtained directly from the rectal ampulla and stored into 200mg aliquots and frozen "in natura" at -20 º C for *nested*-PCR.

The feces were classified according to their consistency into normal (solid consistency) and diarrhea (pasty or liquid). Based on the age range, the animals were allocated to: group 1 (n=91) 5 to 180 days; group 2 (n=102) 181 to 360 days.

Genomic DNA of oocysts was extracted by using the QIAamp DNA Stool Mini Kit® (Qiagen), following the protocol described by the manufacturer, after the sample dilution in ATL buffer and 5 stages of freezing in liquid nitrogen during 1 minute and thawing in Termomix during 3 minutes at 99º C. The DNA was eluted in 50 micro-liters of AE buffer and kept at -20° C.

Molecular characterisation of *Cryptosporidium* spp. was carried out by means of *nested*-PCR for amplification of partial fragments of the subunit 18S rRNA (Xiao *et al*., 2000), followed by sequencing of amplified fragments.

Positive and negative controls for both reactions were genomic DNA from *Cryptosporidium galli* and ultrapure water, respectively.

The samples that had intense amplification of the DNA fragment were purified using the kit QIAquick Gel Extraction® (Qiagen) and underwent sequencing with the ABI Prism® Dye Terminator Cycling Sequence kit (Applied Biosystems) in the automated sequencer ABI 3730XL (Applied Biosystems). Sequencing reactions were performed in both directions, with primer oligonucleotides of secondary reaction.

To determine the consensus sequence, the Codoncode Ali Version 4.0.1 (CodonCode Corporation Dedham®, MA, USA) software was employed. The consensus sequences were aligned by using the Clustal W (Thompson *et al*., 1997) and BioEdit Editor (Hall, 1999) software, based on the homologous sequences available at *GenBank*.

The association between the occurrence of *Cryptosporidium* spp. and the variables sex, breeds, age range and fecal consistency of the analyzed samples were assessed by the Chi-Square test and/or Fisher's exact test. The adopted significance level was 5%, and for statistical analysis the Statistical Analysis System (Statistical…, 2008) software, version 9.2 (2008) was employed.

RESULTS

Of the 193 samples analyzed through *nested*-PCR, 29 (15%) were positive. Considering this total number, sequencing was possible for 19 samples, due to the small amount of DNA limiting the identification of some samples. The analysis of sequences of the 18S rRNA gene allowed the identification of *C. xiaoi* in 15 samples, *C. ubiquitum* in three and *C. meleagridis* in one sample (Table 1).

All farms had positive animals; *C. xiaoi* was not detected in only one farm, which was composed of animals aged from eleven to twelve months. On the other hand, *C. ubiquitum* and *C. meleagridis* were found in only one sheep farm, where pigeons and cats were present near the food of sheep.

There was no statistical difference for the correlation of positivity with breeds and sex of lambs, p=0.1366 and p=0.6120, respectively. According to Chi-square and/or Fisher's test, there was association between age range (p=0.0107) and fecal consistency (p=0.0010) for *Cryptosporidium* spp. occurrence.

Most lambs infected by *Cryptosporidium* spp. were young, less than 180 days old (Tab. 1) and presented diarrhea (Table 2).

Table 1. Distribution of *Cryptosporidium* species detected by means of *nested*-PCR and sequencing, according to age range.

Age range (days)	Animals	Number Positive nPCR (%)	Species Identification[a]
5 – 180	91	20 (21.9)	*C. xiaoi* (8) *C.ubiquituim* (3)
181 – 360	102	09 (8.8)	*C. xiaoi* (7) *C.meleagridis* (1)
Total	193	29 (15.0)	*C. xiaoi* (15) *C.ubiquituim* (3) *C. meleagridis* (1)

[a] Identification of species by sequencing was successful for 19 of 29 samples.

Table 2. Occurrence of infection by *Cryptosporidium* spp. detected through *nested*-PCR, according to fecal consistency.

Fecal consistency	Number of animals	Number Positive nPCR (%)	P(1)
Normal	156	17 (10.9)	0.0010
Diarrheal	37	12 (32.4)	

(1) χ^2 test

DISCUSSION

In the current study, a 15% occurrence of *Cryptosporidium* spp. in lambs was detected through *nested*-PCR. Higher values such as 77.4% in the United States, 25% in Brazil and 24.5% in Australia were reported by Santín *et al*. (2007), Silva (2007) and Yang *et al*. (2009); but Fiuza *et al*. (2011), in Brazil, found only 1.6% positivity for the examined animals. However, Robertson *et al*. (2010), in Norway, evidenced findings similar to ours. Despite all these studies having used molecular techniques, lambs of different ages were surveyed in various countries under different environmental conditions, being influenced by different factors

in epidemiological research of the occurrence of infection with this parasite, which prevents drawing comparisons with the data obtained in our study.

Considering the samples positive for *Cryptosporidium*, most lambs had feces of solid consistency (Table 2), contrasting to the data of Causapé *et al*. (2002), in Spain, who detected higher positivity for diarrheic animals. Regarding age range, other authors have also reported high occurrence of this protozoan in younger animals (Causapé *et al*., 2002; Santín *et al*., 2007).

The species most frequently observed in lambs was *C. xiaoi*, followed by *C. ubiquitum*; similar data were verified in Australia. In a similar way to our study, other authors detected *C. xiaoi* as the most prevalent species in all samples and *C. ubiquitum* as the most commonly detected in younger lambs (Sweeny *et al*., 2011). The latter species has worldwide distribution, *C. ubiquitum* was isolated from humans (Chalmers *et al*., 2009; Xiao, 2010), but has been found in lambs younger than twelve months (Santín *et al*., 2007; Yang *et al*., 2009; Robertson *et al*., 2010; Wang *et al*., 2010; Sweeny *et al*., 2011).

In the present study *C. ubiquitum* was detected in lambs aged from five days to six months. Swenny *et al*. (2011) isolated *C. ubiquitum* in lambs from two weeks to four months of age; however, Wang *et al*. (2010) found *C. ubiquitum* in all age groups (lambs in pre and post weaning, pregnant ewes and after childbirth), which is of greater detection relevance in the pre weaning and therefore was considered the main species observed in sheep in China. Sporadic cases of this species have been described to affect humans (Soba *et al*., 2006); thus, this species must be considered a zoonotic pathogen (Santín *et al*., 2007).

In Brazil this is the first report of *C. xiaoi* in lambs. For genetically confirmed infection by *C. xiaoi* (previously known as *C. bovis-like*), this new species was named after Dr. Lihua Xiao for his contributions to taxonomy and molecular epidemiology of *Cryptosporidium* species (Fayer and Santín, 2009). This parasite was observed in sheep in the USA (Santín *et al*., 2007), Spain (Navarro-I-Martinez *et al*., 2007), Tunisia (Elwin and Chalmers, 2008), United Kingdom (Mueller-Doblies *et al*., 2008), China (Wang *et al*., 2010), Norway (Robertson *et al*., 2010) and Australia (Sweeny *et al*., 2011). In this study, *C. xiaoi* was detected in two age-range groups and was also found by other authors in sheep aged between 14 and 21 days, and between two and 48 months (Santín *et al*., 2007; Navarro-I-Martinez *et al*., 2007; Elwin and Chalmers, 2008; Mueller-Doblies *et al*., 2008). However, Wang *et al*. (2010) found this species in lambs only.

Of the three analyzed farms, *C. ubiquitum* and *C. meleagridis* were found in one single sheep farm, where pigeons and cats were seen at the moment of sample collection in the storage compartment for the food administered to the herd.

Cryptosporidium meleagridis was initially described affecting turkeys (*Meleagris gallopavo*) in 1955 (Slavin, 1955) and then in several bird species, including domestic pigeons (Qi *et al*., 2011). *Cryptosporidium meleagridis* is the third most common species among men and was already detected in both immunocompetent and immunosuppressed humans (Cama, *et al*., 2008). The presence of *C. meleagridis* in the feces of sheep in this experiment does not mean that there was an infection, since there is the possibility of ingestion of oocysts released by the pigeons present in the environment and their passive elimination in the feces.

CONCLUSION

Infection by *Cryptosporidium* was detected in lambs aged up to one year, with prevalence of *C. xiaoi*; thus, this was the first molecular characterization study of this *Cryptosporidium* species in Brazil. *Cryptosporidium ubiquituim* and *C. meleagridis*, two species with zoonotic potential were also found. The latter may have been observed perhaps because of the presence of birds taking shelter in the compartment in which feed was stored, since these animals showed no signs of infection.

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