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 xiaoii 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

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).
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 (anthropogenic) 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. xiaoii 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 °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 99° 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. xiaoï in 15 samples, C. ubiquitum in three and C. meleagridis in one sample (Table 1).

<table>
<thead>
<tr>
<th>Age range (days)</th>
<th>Animals</th>
<th>Number Positive nPCR (%)</th>
<th>Species Identification*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 – 180</td>
<td>91</td>
<td>20 (21.9)</td>
<td>C. xiaoï (8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C. ubiquitum (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C. xiaoï (7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C. meleagridis (1)</td>
</tr>
<tr>
<td>181 – 360</td>
<td>102</td>
<td>09 (8.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>193</td>
<td>29 (15.0)</td>
<td>C. xiaoï (15)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C. ubiquitum (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C. meleagridis (1)</td>
</tr>
</tbody>
</table>

*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.

<table>
<thead>
<tr>
<th>Fecal consistency</th>
<th>Number of animals</th>
<th>Number Positive nPCR (%)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>156</td>
<td>17 (10.9)</td>
<td>0.0010</td>
</tr>
<tr>
<td>Diarrheal</td>
<td>37</td>
<td>12 (32.4)</td>
<td></td>
</tr>
</tbody>
</table>

*χ² 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 all farms had positive animals; C. xiaoï 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).
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. xiaoii, followed by C. ubiquitum; similar data were verified in Australia. In a similar way to our study, other authors detected C. xiaoii 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. Sweeny 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. xiaoii in lambs. For genetically confirmed infection by C. xiaoii (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. xiaoii 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. xiaoii; thus, this was the first molecular characterization study of this Cryptosporidium species in Brazil. Cryptosporidium ubiquitum 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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Molecular characterisation...


