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Short Communication

Cryptosporidium occurrence in ruminants from the North Pioneer mesoregion of Paraná, Brazil

Ocorrência de *Cryptosporidium* em ruminantes da mesorregião norte pioneiro do Estado do Paraná, Brasil

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Abstract

The aim of this study was to investigate the occurrence of *Cryptosporidium* in cattle and sheep from the North Pioneer mesoregion of the state of Paraná. For this, 317 stool samples were collected from cattle and sheep on 16 properties in six municipalities in the North Pioneer mesoregion of Paraná. For detection of *Cryptosporidium* species, molecular analysis was performed using nested-PCR techniques targeting the 18S rRNA gene. Of the 37 beef cows and 115 calves analyzed, four (10.8%) and 14 (12.2%), respectively, were positive for *Cryptosporidium*. Of the 12 cows and 52 calves, one (8.3%) and 14 (26.9%), respectively, were positive for *Cryptosporidium*; and of the 42 ewes and 59 lambs, six (14.3%) and 12 (20.3%), respectively were positive for *Cryptosporidium*. Cattle (15.3%) and sheep (17.8%) were both susceptible to infection. All the properties of the municipalities of Assaí, Ibaiti and, Leópolis presented infected animals. The study showed that *Cryptosporidium* occurs in most municipalities assessed, that dairy calves had a higher risk (Odds Ratio=2,66, *p-value*=0,018) for infection than beef calves, and that sheep are just as susceptible to infection as are cattle, and that further *Cryptosporidium* studies are developed.

Keywords: Cryptosporidiosis, cattle, sheep, occurrence, Southern Brazil.

Resumo

O objetivo deste estudo foi investigar a ocorrência de *Cryptosporidium* em bovinos e ovinos da mesorregião norte pioneiro do Estado do Paraná. Para tanto, 317 amostras de fezes destes ruminantes foram colhidas de 16 propriedades de seis municípios do Norte Pioneiro do Paraná. Para detecção de *Cryptosporidium* spp foi realizada análise molecular pela Técnica de nested-PCR direcionada ao gene 18S rRNA. Das 37 vacas de corte e 115 bezerros de corte analisados, quatro (10,8%) e 14 (12,2%) foram respectivamente positivos para *Cryptosporidium*. Das 12 vacas e 52 bezerros de leite, um (8,3%) e 14 (26,9%) foram positivos para *Cryptosporidium* e das 42 ovelhas e 59 cordeiros avaliados, seis (14,3%) e 12 (20,3%) amostras estavam positivas para *Cryptosporidium*, respectivamente. Bovinos (15,3%) e ovinos (17,8%) foram igualmente suscetíveis à infecção. Todas as propriedades dos municípios de Assaí, Ibaiti e Leópolis apresentaram animais infectados. Este estudo demonstrou que *Cryptosporidium* ocorre na maioria dos municípios avaliados, sendo que os bezerros de leite apresentam maior risco (Razão de chances=2,66, *p-value*=0,018) à infecção que os bezerros de corte e que os ovinos são tão suscetíveis à infecção quanto os bovinos e por isso, estudos nesta espécie animal devem ser mais desenvolvidos.

Palavras-chave: Cryptosporidiose, bovinos, ovinos, ocorrência, Sul do Brasil.

Cryptosporidium is a genus of apicomplexan protozoan from the class Conoidasida, order Eucoccidiorida, and family Cryptosporidiidae; *Cryptosporidium* species have a life cycle that is primarily completed within the gastrointestinal tract of a single host (LEVINE, 1985). However, new evidence suggests that the *Cryptosporidium* genus is included in the subclass Gregarina (CLODE et al, 2015).

In ruminants, the occurrence of *Cryptosporidium* has been linked to gastroenteritis in calves between 0 and 2 months of age and a high morbidity rate in dairy cattle (COKLIN et al., 2007; SANTÍN et al., 2008), calves prior to weaning and in newly weaned

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calves (AYINMODE et al., 2010; FAYER et al., 2008), and in older cattle (KVÁC & VITOVEC, 2003; ROBINSON et al., 2006). In sheep, the highest infection rates are observed in the first few weeks of life (MARTINEZ & BELDA, 2001), with increased infection rates up to 21 days (SANTÍN et al., 2007). Older lambs demonstrate delayed growth, and adults are generally refractory to infection and illness (XIAO et al., 1993), but can eliminate oocysts in the feces, thereby contaminating the environment (MAJEWSKA et al., 2000; ZUCATTO et al., 2015).

In Brazil, studies on cryptosporidiosis in ruminants have primarily been performed in the Southeast, mainly in the states of Rio de Janeiro (COSENDEY et al., 2008; FIUZA et al., 2011), Minas Gerais (GARCIA & LIMA, 1993; GARCIA & LIMA, 1994; LIMA et al., 2013), and São Paulo (AQUINO et al., 2015; COELHO et al., 2016; FEITOSA et al., 2004; FÉRES et al., 2009; PAZ E SILVA et al., 2013, 2014; SEVÁ et al., 2010; SILVA-JUNIOR et al., 2011). In the state of Paraná, infection with *Cryptosporidium* was evaluated in captive birds (NAKAMURA et al., 2009), dogs with diarrhea (NAVARRO et al., 1997), horses (FUJII et al., 2014), wild birds and mammals (SNAK et al., 2015), cattle (TOLEDO et al., 2017) and sheep (SNAK et al., 2017). However, epidemiological studies have not been conducted on cryptosporidiosis in ruminants of the north pioneer mesoregion of Paraná. Knowledge about cryptosporidiosis among ruminants in this region of Paraná may have important implications in the field of veterinary public health, since this disease is a zoonosis. When results of this type of study are published, health surveillance agencies and other health professionals can alert the local population about hygiene measures necessary for the prophylaxis of the disease in animals and people living in the area. Thus, the present study aimed to evaluate *Cryptosporidium* infection and to determine its frequency of occurrence among adult and young cattle, dairy cows and adult and young sheep of the region.

Fecal samples of 317 ruminants who were apparently healthy, without symptoms of diarrhea, from 16 farms were chosen at random from six municipalities of North Pioneer, as follows: 75 samples from two farms in the municipality of Assaí, 63 samples from six farms in the municipality of Santo Antonio da Platina, 44 samples from two farms in the municipality of Ibaiti, 48 samples from three farms in the municipality of Ribeirão do Pinhal, 20 samples from one farm in the municipality of Ribeirão Claro, and 67 samples from two farms in the municipality of Leópolis. The period of sample collection was from August 2013 to May 2014. Of the 317 samples collected, 115 were from beef calves (aged ≤ 7 months), 52 from dairy calves (aged ≤ 6 months), 37 beef cows (aged > 3 years), 12 dairy cows (aged > 3 years), 59 from lambs (aged ≤ 4 months), and 42 from ewes (aged > 1.5 years). The collected samples were transported in a refrigerated condition to the Laboratory of Parasitology and Parasitic Diseases of the State University of North of Paraná; and an aliquot of each sample was separated and frozen until DNA extraction.

DNA extraction was performed using a commercial kit—NucleoSpin® Tissue (Macherey-Nagel GmbH & Co.)—and following the manufacturer's protocol, with three incremental freeze and thaw cycles before the lysis step in order to improve the rupture of oocysts (WELLS et al., 2015).

For the detection of *Cryptosporidium* species, a 826-840 bp fragment of the 18S ribosomal RNA (rRNA) gene was amplified by nested polymerase chain reaction (PCR) with primers (XIAO et al., 1999).

Negative controls consisting of ultrapure water and positive controls consisting of *C. parvum* DNA were used in all batches. The products were subjected to 1.5% agarose gel electrophoresis, stained with SYBR Safe® (Invitrogen Co.), and photographically documented.

A descriptive variable analysis (positive/negative) was performed for *Cryptosporidium* infection in the populations of cattle and sheep, as well as for determining the odds ratio and their respective confidence intervals for the exposure variable (age, type, and category) and outcome (infection). The association between risks was assessed using Fisher's exact test, with significance set at *p-value*< 0.05. Statistical analyses were performed using GraphPad Prism 6.01 (GraphPad Software, Inc.).

This project was approved (CEUA 3164-48) by the Comitê de Ética do Uso de Animais (CEUA), Universidade Estadual do Norte do Paraná.

Of the total 317 samples tested, 51 (16.1%) were positive for *Cryptosporidium*. Of the 37 beef cows and 115 calves analyzed, four (10.8%) and 14 (12.2%), respectively, were positive for *Cryptosporidium*. Of the 12 cows and 52 calves analyzed, one (8.3%) and 14 (26.9%), respectively, were positive for *Cryptosporidium*; and of the 42 ewes and 59 lambs, six (14.3%) and 12 (20.3%), respectively, were positive for *Cryptosporidium*. However, statistically significant differences were found only between beef cattle and dairy cattle. These differences were related to higher infection rates among dairy calves (26.9%) than among beef calves (12.2%), *p-value*=0.018. Dairy calves were 2.7 times more likely to be infected with *Cryptosporidium* that were beef calves (OR = 2.66; CI 1.16-6.10%)

A similar infection rate (28.1%) was reported in dairy calves in Canada by using the Sheather's flotation method (COKLIN et al., 2007), but other authors have found higher occurrence rates of *Cryptosporidium* (43.6–82.1%) using the modified Ziehl-Neelsen method (EDERLI et al., 2004), and Sheather's or Ziehl-Neelsen methods followed by PCR techniques (COUTO et al., 2014; TOLEDO et al., 2017). Of the 216 cattle evaluated, 33 (15.3%) were positive for *Cryptosporidium,* as were 18 (17.8%) of the 101 sheep. Although the rate of infection was higher in sheep, this difference was not significant (*p-value* = 0.57) (Table 1). Contrary to the results of this study, Villacorta et al. (1991) analyzed 141 cattle and 69 sheep and found infection rates of 6.38% and 1.45% respectively, using Ritchie's Technique modified by Allen & Ridley (1970).

Although not significant (*p-value* = 1.15) there were more positive lambs (20.3%) than beef calves (12.2%). While comparative studies are scarce, an earlier study of 460 sheep in Araçatuba, São Paulo showed a 6.7% positive rate for oocysts using Sheather's flotation method, which is in agreement with our findings (FÉRES et al., 2009). In the same city, 12.4% of the calves were found to be positive using this same technique in a similar number of samples (FEITOSA et al., 2004). In the State of Paraná, recent research in the Western region showed high positivity in adult sheep (59%), however the authors evaluated few lambs (7/144) (SNAK et al., 2017)

The infection rate among lambs was 43% higher than that seen in sheep. Although this difference was not significant, these

Table 1. Distribution of positive and negative animals, relative frequency (%), and statistical analyzes (OR - Odds Ratio, CI - Confidence Interval, p-value and χ^2-Chi-square) of results from fecal tests for *Cryptosporidium* infection in adult and young cattle and sheep in the North Pioneer mesoregion of Paraná, Brazil.

Variable	**Infection results**		**% Relative**	**OR (CI)**	**p-value**	**χ^2**
Animal category	**Positive**	**Negative**				
Beef cow	4	33	10.8	1.33 (0.13-13.2)	0.805	0.06
Dairy cow	1	11	8.3			
Species						
Cattle	33	183	15.3	1.20 (0.44-1.56)	0.57	0.33
Sheep	18	83	17.8			
Age						
Young	40	186	17.7	1.56 (0.76-3.2)	0.22	1.51
Adult	11	80	12.1			
Category/Specie/Age						
Lamb	12	47	20.3	1.53 (0.52-4.47)	0.43	0.61
Ewe	6	36	14.3			
Calf	28	139	16.8	1.77 (0.65-4.87)	0.26	1.26
Cow	5	44	10.2			
Beef calf	14	101	12.2	**2.66* (1.16-6.10)**	0.018	5.58
Dairy calf	14	38	26.9			
Beef cow	4	33	10.8	1.33 (0.13-13.24)	0.8	0.06
Dairy cow	1	11	8.3			
Beef calf	14	101	12.2	1.84 (0.79-4.29)	1.15	2.05
Lamb	12	47	20.3			
Dairy calf	14	38	26.9	1.44 (0.59-3.48)	0.65	0.21
Lamb	12	47	20.3			
Beef cow	4	33	10.8	1.38 (0.35-5.31)	0.64	0.22
Ewe	6	36	14.3			
Dairy cow	1	11	8.3	1.83 (0.19-16.92)	0.59	0.29
Ewe	6	36	14.3			

*Significate difference ($p < 0.05$)

results were similar to those published by Causapé et al. (2002) and Paz & Silva et al. (2014).

The variation in the infection rate, as determined by the presence of oocysts in the feces of animals infected with *Cryptosporidium*, is probably because of the form of laboratory diagnosis (for direct or molecular parasitological techniques) and also the clinical condition of the animals.

Techniques such as the modified Ziehl-Neelsen method, direct fluorescent antibody test, negative staining method or ELISA, with or without subsequent PCR, are widely used in the study of cryptosporidosis in ruminants. Among these techniques, PCR has the highest sensitivity (MORGAN et al., 1998; REKHA et al., 2016; TAHVILDAR-BIDEROUNI & SALEHI, 2014); moreover, the reported rates of occurrence of *Cryptosporidium* vary between studies (CAUSAPÉ et al., 2002; EDERLI, et al., 2004; FÉRES et al., 2009; PAZ & SILVA et al., 2014; SNAK et al., 2017; TOLEDO et al., 2017). Higher infection rates are reported with the use of a stool oocyst-concentration technique, such as Sheather's flotation method (COKLIN et al., 2007; COUTO et al., 2014), probably due to its high sensitivity (REKHA et al., 2016). The discrepancy of sensitivity and specificity between the diagnostic techniques for *Cryptosporidium* occurrence indicates the need for multiple laboratory techniques for better reliability of the results.

Table 2. Distribution of the total *Cryptosporidium* infection rates of beef calves, dairy calves, beef cows, dairy cows, lambs, and ewes for the municipalities of Assaí, Santo Antônio da Platina (Sto A Platina), Ibaiti, Ribeirão do Pinhal (Rib Pinhal), Ribeirão Claro (Rib Claro), and Leópolis in the state of Paraná, Brazil.

Municipalities	Total (%)	Beef calves (%)	Dairy calves (%)	Beef cows (%)	Dairy cows (%)	Lambs (%)	Ewes (%)
Assai	18.7	16.0	21.4	14.3	0	33.3	—
Sto A Platina	11.1	18.8	11.1	0	—	11.1	9.1
Ibaiti	36.4	33.3	46.7	33.3	16.7	—	37.5
Rib Pinhal	10.4	8.7	—	14.3	—	0	18.2
Rib Claro	0	0	—	0	—	0	—
Leópolis	13.4	8.0	—	0	—	29.2	0

The present study did not used diarrheic samples. This may be one reason the infection rate found in this study was lower than that reported by several other authors, including Causapé et al. (2002), that analyzed 1-week-old lambs with diarrhea and found that 93.3% of the animals were positive for *Cryptosporidium* infection, as determined by the Ziehl-Neelsen technique. Ayinmode et al. (2010) reported that 52.3% of PCR samples from calves with diarrhea in Nigeria were positive for *Cryptosporidium* infection and Garcia & Lima (1994) found almost 60% of the calves positive for *Cryptosporidium* infection as per the Ziehl-Neelsen technique had diarrhea. Other studies in ruminants—regardless of the presence of diarrhea—have shown infection rates lower (0-12.4%) than those reported in the present study (COKLIN et al., 2007; FEITOSA et al., 2004; FÉRES et al., 2009; CARDOSO et al., 2008).

Young animals are expected to have a higher risk for infection, as compared to the adults; however, our results indicated no difference in the occurrence of *Cryptosporidium* between calves (16.8%) and cows (10.2%), possibly due to the similar infection rates observed in calves and cows (12.2 and 10.8%, respectively) or the low number of cows analyzed (n = 12). Beef cows and calves live in the same environment, which possibly accounts for the similar infection rates between these sub-groups. Genotypic analyses would be necessary to confirm this hypothesis, since distinct species of *Cryptosporidium* infect animals of different ages. Dairy calves are separated from their mothers, and usually maintained under unhygienic conditions, which could explain the trend toward higher occurrence rates in calves than in milk cows. In previous comparative studies, Coklin et al. (2007) evaluated 143 dairy cows and found an infection rate of zero (0%) in cows and 39.6% in calves. Cardoso et al. (2008) reported *Cryptosporidium* infection rates of 1.8% in calves (aged 0-6 months) vs. 0.35% in cows and heifers; and Gow & Waldner (2006) reported 1.1% (5/560) in beef cows and 3.1% (19/605) in beef calves.

The similar rate of occurrence of *Cryptosporidium* in young and adult animals found in this study could also be explained by a high infection rate in adult cattle by *C. andersoni*. This species is found in animals from 1 year of age to older animals (up to 2 years old), often asymptomatic bearers of environmental contamination (ROBINSON et al., 2006)

Cryptosporidium infections in beef cattle are not frequent (HECKLER et al., 2015). In Brazil, Cardoso et al. (2008) found only 0.4% of cows to be positive for *Cryptosporidium* in the municipality of Caçapava, São Paulo. Ralston et al. (2003), and Gow & Waldner (2006) also identified a low prevalence of *Cryptosporidium* in calves and were among the few researchers to evaluate beef cattle.

Of the 16 properties studied, 11 (68.7%) had at least one animal that was positive for *Cryptosporidium*. All properties in the municipalities of Assaí (2/2), Ibaiti (2/2), and Leópolis (2/2) had infected animals. Half of the Santo Antônio da Platina properties (50%) and 66.7% of the Ribeirão do Pinhal properties had positive animals (Table 2). Epidemiological studies of parasitic diseases in the North Pioneer mesoregion are scarce. However, the few studies on ruminants in this region have shown high parasite loads, which may reflect poor sanitary conditions and inadequate handling of these animals (HOLSBACK et al., 2013, 2015, 2016).

It was concluded that *Cryptosporidium* occurs in most municipalities assessed, that dairy calves have a higher risk for infection than beef calves, and that sheep are just as susceptible to infection as cattle. This was the first epidemiological study of cryptosporidiosis in ruminants in the North Pioneer mesoregion of Paraná.

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