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Cryptosporidium infection in diarrheal bovine dairy calves: occurrence and risk factors in Santa Catarina, Brazil

Infecção por *Cryptosporidium* em bezerros com diarreia: ocorrência e fatores de risco em Santa Catarina, Brasil

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Highlights

High prevalence of diarrhea in calves caused by *Cryptosporidium* spp.
Cryptosporidium spp. in calves in Santa Catarina, Brazil.
Importance of molecular characterization of *Cryptosporidium* spp. in stool samples.
Coproparasitological and molecular detection of *Cryptosporidium* spp. in fecal samples.

Abstract

Cryptosporidium protozoa genus are parasites that cause acute enteric disease in young and immunocompromised animals, resulting in anorexia, loss and decrease in weight gain, and, in severe cases, death. Therefore, this study aimed: i) to determine the occurrence of *Cryptosporidium* spp. in calves with clinical diarrhea in different regions of Santa Catarina, Brazil; ii) to evaluate the risk factors involved with the frequency of infection. iii) to determine the species most involved with the disease in the region. For this, 425 samples were collected in 141 dairy farms, from animals with ages ranging from 0 to 150 days. For this

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purpose, the samples were submitted to the modified Ziehl-Neelsen technique, with molecular analysis of the positive samples being performed. It was observed 62.1% occurrence of *Cryptosporidium* spp. in this sampling, especially between 8 to 15 days. Regarding the risk factors evaluated, such as age, management, facilities, water source and Koppen climate (CFA and CFB), none showed statistical significance. Samples positive by the Ziehl-Neelsen technique (32 samples) were randomly selected for molecular diagnosis. Of these, 10 were sequenced, allowing the identification of *Crypstosporidium parvum* in 6 samples. However, this study proves the existence and high occurrence of the protozoan in different regions of the state of Santa Catarina, Brazil.
Key words: Claw integrity. Lameness. Parity order. Sow longevity.

Resumo

Os protozoários do gênero *Cryptosporidium* são parasitas que causam doença entérica aguda em animais jovens e imunocomprometidos, resultando em anorexia, perda e diminuição do ganho de peso e, em casos graves, morte. Portanto, este estudo teve como objetivo determinar a ocorrência de *Cryptosporidium* spp. em bezerros com diarreia clínica em diferentes regiões de Santa Catarina, Brasil; bem como avaliar os fatores de risco envolvidos com a frequência de infecção. Além disso, com um número seleto de amostras, buscou-se determinar as espécies mais envolvidas com a doença na região por meio de técnicas moleculares. Para isso, foram coletadas 425 amostras em 141 fazendas leiteiras, de animais com idade variando de 0 a 150 dias. Observou-se 62,1% de ocorrência de *Cryptosporidium* spp. nesta amostragem, principalmente entre 8 a 15 dias. Em relação aos fatores de risco avaliados, como idade, manejo, instalações, fonte hídrica e clima de Koppen (CFA e CFB), nenhum apresentou significância estatística. No entanto, este estudo comprova a existência e alta ocorrência do protozoário em diferentes regiões do estado de Santa Catarina, Brasil.
Palavras-chave: Criptosporidiose. Gado leiteiro. Epidemiologia. Protozoário. Ziehl Neelsen.

Introduction

Cryptosporidiosis, caused by a protozoan of the genus *Cryptosporidium*, is an important neonatal disease in dairy calves, capable of causing diarrhea and intense dehydration (Shaw et al., 2021). Although in many cases it is considered a self-limiting disease (Thomson et al., 2017), in long term, the damage caused by cryptosporidiosis may be irreversible and continue into the adult life of the animals, directly impacting milk production (Shaw et al., 2020) and may change the region's production rates. In 2020, the Brazilian state of Santa Catarina, which was considered the 4th largest milk producer in Brazil, produced 3,137 million liters of milk, corresponding to 11.6% of national production (Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina [EPAGRI], 2022).

In Brazilian territory, it is described to have 51.52% of occurrence in calves (Cruvinel et al., 2020), while Oliveira et al. (2021) demonstrate 50% of occurrence for the species *C. parvum.* In cattle, there are four important species, being *C. parvum,* which affects newborn calves between one

to three weeks; *C. bovis* and *C. rynae,* which infect young animals, between 3 to 8 months; and *C. andersoni,* which parasitizes older animals (Budu-Amoako et al., 2011; Díaz et al., 2018; Gong et al., 2017; Ryan & Hijjawi, 2015). Since cattle can shed up to 100,000 oocysts daily through feces, they are considered the biggest spreaders of the disease today (Nader et al., 2019). Therefore, it is essential to control the disease in cattle farms, especially by observing the risk factors involved, such as age, facilities, water source and production system (García-Romo et al., 2014).

Even after 100 years of its discovery, cryptosporidiosis remains one of the most difficult diseases to control in the herd (Thomson et al., 2017) possibly due to the fact that in determined regions, there is little information about the occurrence and risk factors of the disease in neonates., making control and prevention even more difficult. Environmental control becomes difficult especially due to the size of the oocysts, which ranges from 4 to 6μm, in addition to survival for months in the environment (Nugraha et al., 2021). However, control measures must be worked out, because they affect so much animal health, bringing countless losses to the industry; and putting the health of millions of people at risk (Brainard et al., 2020). Environmental control measures involving cryptosporidiosis are difficult because oocysts are resistant to disinfectants (Thomson et al., 2017).

Thus, considering characteristics such as monoxene cycle, possible zoonotic transmission, prolonged environmental survival, a low infective dose associated with high excretion, in addition to extremely difficult control and treatment (Lal et al., 2013), combined with the scarcity of global and regional studies, this work was developed to determine the occurrence of *Cryptosporidium* spp. and possible species in diarrhea samples, as well as to evaluate the risk factors involved with infection by the protozoan.

Material and Methods

Study site and sampling

The study was conducted on dairy-based economy farms, including the regions of Santa Catarina, except for the coastal region. A total of 425 samples were collected from 141 farms, which included the five mesoregions of Santa Catarina, Brazil. The number of samples were calculated using the OpenEpi software 3.01, with 50% prevalence, 5% expected error, and 95% confidence interval (CI). The number of samples per region was based on the total population, error margin, a number of standard deviations between a given proportion, and the mean at a 95% confidence level. This study included calves aged between 0 and 150 days with signs of diarrhea. Fecal samples were collected directly from the rectum of animals using gloves, stored in isothermal boxes, and sent to the laboratory. The collections were conducted from March to August 2019. The present study was approved by the Animal Ethics Committee (AEC) of the Universidade do Oeste Catarinense (Unoesc - Xanxerê), protocol number 57/2018.

Parasitological analysis

The parasitological analysis of feces was performed at the Laboratório de Parasitologia Veterinária of Unoesc. For this, a stool smear was made on each of the slides, and then an additional smear from the Sheather technique supernatant was carried out. Modified Ziehl-Neelsen technique was used to stain the slides (ZIEHL NEELSEN staining set, Laborclin®) (Feitosa et al., 2004; Molina et al., 2009). A Nikon optical microscope (E200) was used to visualize the slides under 1000× magnification using immersion oil. This procedure provides an increase in the sensitivity of the techniques due to the concentration of oocysts.

Molecular analysis

For this purpose, 32 positive samples by Ziehl-Neelsen technique were previously randomly selected for molecular diagnosis. About 5 g of homogenized feces were added to 10 ml of distilled water was sieved, and the solution was transferred to 15 ml conical tubes and centrifuged at 2000 rpm for 5 min for sedimentation. The sediment was stored at -80°C and sent to the Laboratório de Doenças Parasitárias, Universidade Federal de Santa Maria.

Total DNA was extracted using the PureLink® Genomic DNA Mini Kit (Invitrogen, USA), with approximately 200 mg of each fecal sample as per the manufacturer's instructions. The DNA samples were stored at -20°C for molecular analysis.

To detect *Cryptosporidium* spp., the total DNA (approximately 200 ng) of each sample was analyzed using nested-PCR to amplify the sequence of the SSU rRNA gene using the following primer pairs: 1. Primary PCR: Forward (F1) 5′-TTCTAGAGCTAATACATGCG-3′ and reverse (R1) 5′-CCATTTTCGAAACAGGA-3′; 2. Secondary PCR: Forward (F2) 5′-GGAAGGTTATTTAGATAAAG-3′ and reverse (R2) 5′-AGGAGTAAGGAACAACCTCCA-3′, resulting in product sizes of 1325 bp and 819-825 bp (depending on the species), respectively (Alves et al., 2018).

Primary PCR was performed in a reaction volume of total volume of 25 µl containing 3 µl DNA (100 to 200 ng total DNA), 0.5 µM of each primer, 2.5 mM MgCl2, 10 mM dNTPs, 1× reaction buffer, and four units of Taq polymerase (Invitrogen®), and MilliQ water. The conditions for secondary PCR were the same as for primary PCR, except for the use of a different pair of primers and 2 µl of the first PCR amplification product as a mold in a final reaction volume of 25 µl. *Cryptosporidium parvum* genomic DNA was used as a positive control and MilliQ water was used as a negative control. The PCR conditions were as follows: initial denaturation at 94°C for 3 min; followed by 40 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min; and a final extension at 72°C for 7 min. The conditions were the same for both reactions, except that in the second reaction, 35 cycles were used and it was performed in a T100™ Thermalcycler (Bio-Rad, Singapore). The PCR products were analyzed in 1.5% agarose gel stained with DimondTM Nucleic Acid Dye Promega and visualized under ultraviolet light after electrophoresis (60V, 60 min).

Ten PCR amplified products were purified using the QIAquick PCR Purification Kit® (Qiagen™, Germany), according to the

manufacturer's instructions. Final purified DNA was analyzed using spectrophotometer NanoDrop 1000 (ThermoScientific, USA) for concentration determination. After purification, the sequencing reactions were performed using 5 pmol of primers separately, 50 ng of purified PCR product and MiliQ water in a final volume of 6 µl. Followed by dehydration at 50 ºC for 1 hours and finally submitted to sequencing in quadruplicate using the Sanger method (ACTGENE - Sequencing Service, Brazil). The results were analyzed using the Staden Package software (http://staden.sourceforge.net/), and the generated nucleotide sequences were evaluated using BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

Epidemiological data form

The questionnaire was applied by previously trained technicians on commercial properties. The questions were age, management, facilities and water source.

Statistical analysis

Univariate analysis by logistic regression was performed for each risk factor on the dependent variable (positive or negative for parasites) and the *odds ratio* (OR) and its CI (95%) were estimated for each effect considered in the model: age, management, facilities, water source, and Köppen climate classification (Köppen, 1901). The R software (Team, 2013) was used with significance levels of 5% ($p<0.05$).

Results and Discussion

The microscopic analysis revealed detection of 62.1% (164/264) of *Cryptosporidium* spp. oocysts. Despite this, the majority (61.8%- 207/264) of the samples were collected in the western region. (Figure 1).

Among the 32 samples analyzed by molecular techniques, 10 samples were sent for Sanger sequencing. Of these, 6 samples were characterized as *C. parvum* and 4 samples could not be characterized.

Through univariate logistic analysis, some risk factors were evaluated, such as age, rearing system, facilities, water source and climate (Table 1). As a result, not all properties contained all the information present in the questionnaire. As for age, most of the positive samples were concentrated in ages between 8 and 15 days (21.48%). Regarding management, the highest concentration of positive samples is related to intensive rearing management (31.59%) and individual installations (35.9%). When analyzing the water source of the property, it was possible to verify that the highest occurrence is concentrated in animals that receive water from underground (41.71%). Furthermore, regarding climate, there was no significant difference between CFA and CFB, showing similar results for both.

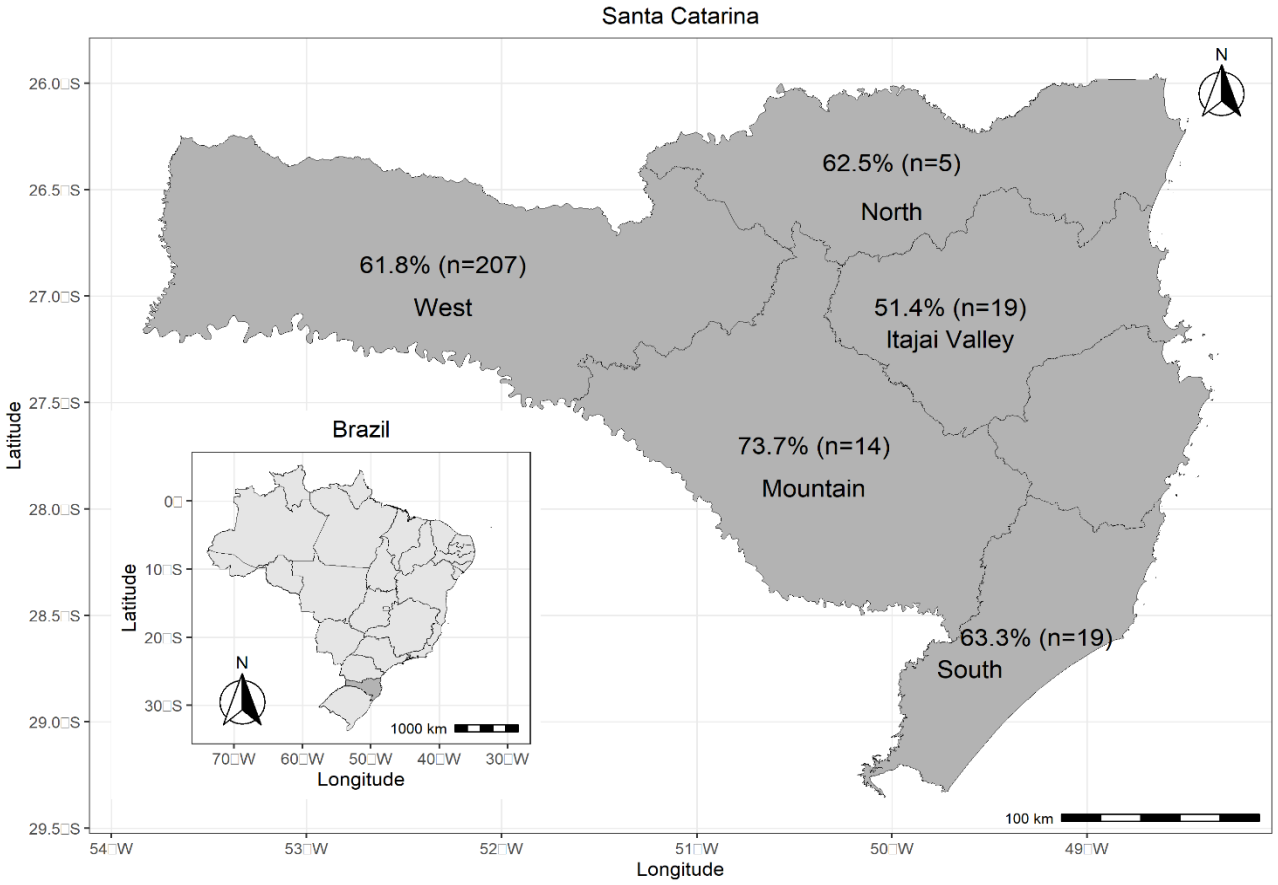

Figure 1. Map of Santa Catarina state, Brazil, showing the main regions where feces samples were collected from calves to evaluate the occurrence of *Cryptosporidium* spp.

This protozoan has a worldwide distribution, covering between 3.4-96.6% of animals, according to Thomson et al. (2017). *Cryptosporidium* spp. is the main diarrhea causative agent., either alone or in co-infections. In Brazil, there is a discrepancy between the data obtained in the present work and the other studies found, especially due to age variation, in addition to the techniques used in diagnosis, farm management and laboratory characteristics. Almeida et al. (2010) found 61% of occurrence in cattle over 12 months of age, in Rio de Janeiro, with or without clinical signs of diarrhea. In the state of São Paulo, there was an 11.5% occurrence in animals over 30 days of age, with only 30% of the animals showing clinical signs (Feitosa et al., 2004). With results similar to the present study, Cruvinel et al. (2020) found a 50% prevalence of *Crypstosporidium* spp. in Xanxerê, Santa Catarina. In a recent study carried out in some Brazilian states, it was revealed that 52.4% of the samples were positive for *Cryptosporidium* spp., finding a percentage of 100% for *C. parvum,* among the select number of samples processed (Oliveira et al., 2021). In the present study, 32 samples were positive in the molecular

analysis, and 10 samples were sent for sequencing. Of these, 6 samples were characterized as *C. parvum.* However, 4 samples could not be characterized. Although PCR is still an excellent tool today, molecular characterization may have been limited by several factors, including the quantity and quality of the DNA preparation and the presence of PCR inhibitors in the animal's feces (Bakheit et al., 2008).

Table 1
Univariate logistic regression analysis of factors associated with *Cryptosporidium* spp. infection in cattle in the state of Santa Catarina, Brazil

Variables		N (+)	N (-)	% (+/ total)	OR	IC -95%	p-valor
Age (days)	0 - 7	37	18	9.14			0.181
	8 - 15	87	55	21.48	0.77	0.40-1.48	
	16 - 23	34	22	8.40	0.75	0.35-1.64	
	24 - 31	37	25	9.14	0.72	0.34-1.54	
	32 - 39	9	10	2.22	0.44	0.15-1.27	
	40 - 47	22	10	5.43	1.07	0.42-2.73	
	48 - 55	5	1	1.23	2.43	0.26-22.39	
	56 - 63	12	2	2.96	2.92	0.59-14.45	
	64 - 71	2	2	0.49	0.49	0.06-3.74	
	> 72	13	2	3.21	3.16	0.64-15.53	
Management	Intensive	127	69	31.59			0.606
	Semi-intensive	90	52	22.39	0.94	0.60-1.47	
	Extensive	37	27	9.20	0.74	0.42-1.32	
Facilities	Individual	135	60	35.90			0.186
	Collectives and closed	80	57	21.28	0.62	0.40-0.98	
	Collectives and pickets	18	9	4.79	0.89	0.38-2.09	
	Individual and pickets	10	2	2.66	2.22	0.47-10.45	
	Extensives	3	2	0.80	0.67	0.11-4.09	
Water source	Underground water	166	92	41.71			0.565
	Artesian well (pressurizad)	86	54	21.61	0.88	0.58-1.35	
Koppen	CFA	186	118	61.18			0.814
	CFB	78	47	62.40	1.05	0.69-1.62	

Oocyst excretion begins between 2 and 6 days of age (Uga et al., 2000), reaching peak excretion in the second week, with a tendency to decline in subsequent weeks (Santín et al., 2008). This information corroborates the present study, which demonstrates a higher percentage of excretion at 8 to 15 days of age. Similar information was found in a study by García-Romo (2014), where 81% of animals aged

between 8 and 14 days showed positivity. In addition to this fact, Nydam et al. (2001) states that a calf is capable of excreting millions of oocysts daily, depending on its age. Animals between 6 and 12 days old are capable of excreting up to $3.86x10^{10}$, while an cattle aged between 50 to 56 days tends to decrease excretion significantly up to $3.8x10^{7}$. Despite this, in most cases, depending on the species involved, diarrhea is not associated with the number of oocysts excreted (Åberg et al., 2019). The small amount of oocysts needed to trigger the disease should also be taken into account, together with the parasite's high capacity for replication, which directly impacts environmental contamination and subsequent difficulty in control and treatment (Thomson et al., 2017).

In addition to the age factor, the clinical severity of cryptosporidiosis is directly linked to the immunological and nutritional status of the animal (Ayele et al., 2018), with colostrum being the main nutritional source of newborn animals. However, if there is no adequate storage, as well as optimal thawing, the effectiveness of colostrum can be compromised. In addition, in the case of cryptosporidiosis, animals that ingest colostrum from banks are at great risk of infection (Brainard et al., 2020), despite the discrepant genetic diversity between adult and juvenile animals (Shaw et al., 2021). Ideally, calves should ingest colostrum (10% of their live weight) in the first 6 h of life because after that period, the capacity to absorb immunoglobulins is reduced (Bessi et al., 2002). Silva et al. (2011) observed that calves receiving colostrum within 6 h of birth had a lower occurrence of *Cryptosporidium* spp. infection.

According to a study by Urie et al. (2018), when the ambient temperature exceeds the animal's comfort temperature, there are greater risks of infection, which is linked to the fact that heat stress directly influences the animal's immunity. Therefore, it is evidenced by Trotz-Williams et al. (2007) that the hot climate can be considered a risk factor for infection to animals. In the state of Santa Catarina, which provides a humid subtropical and oceanic climate, the protozoan will be able to maintain in the environment. Despite this, the results of the analysis through Koppen (CFA and CFB) were not significant, in addition to being very similar. In addition to the regional climate, the Brazilian territory is considered one of the hotspots for the transmission of cryptosporidiosis via water, taking into account that the protozoan can travel, through rivers, for kilometers of distance, until they sediment and penetrate the deeper layers of the waters and remain infectious for months (Vermeulen et al., 2019). In addition to this factor, rainy periods that culminate in flooding directly impact environmental contamination and dissemination by oocysts (Lal et al., 2013).

In this study, the highest occurrence of infection was observed in animals housed in individual pens. It is noteworthy that most of the animals sampled with clinical signs of diarrhea were in the age range of 8-15 days, a period in which the calves are housed in individual pens. This is indicative of the greater occurrence associated with this form of rearing. Individual pens are used by many producers to avoid disease transmission among animals by direct contact. The rate of infection observed in this study in individually reared animals may be determined by the

lack of hygiene in a facility previously used by a possible infected animal. The continuous use of the same facility without previous disinfection facilitates the survival of the agent in the environment and the subsequent infection of the next animal because adequate temperature and humidity conditions favor the viability of oocysts (Fayer et al., 2000). In addition, the contamination of equipment and food and water supply sites with protozoan oocysts eliminated by infected animals facilitates the spread of *Cryptosporidium* spp. because the transmission route is fecal-oral (Almeida et al., 2010). However, other studies show that animals housed in collective pen facilities have the highest occurrence of infection, with variations of up to 80.5% and related to dependent age (Åberg et al., 2019; Feitosa et al., 2004; Silva et al., 2011). In addition to age, these differences between individual and collective facilities may be related to high population density, which consequently increases the risk of infection owing to the greater contact of healthy animals with carriers (Sturdee et al., 2003).

Conclusions

The present study demonstrated a high prevalence of diarrhea in calves caused by *Cryptosporidium* spp. in different regions of the state of Santa Catarina, with evidence of *C. parvum* in some samples. Due to the zoonotic potential of the nature of this parasite, more molecular studies should be used in the region, especially to recognize subtypes harmful to humans, as well as monitor new species and increase the sensitivity in the diagnosis, identifying possible sources of contamination.

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Declarations

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Ethics approval

The present study was approved by the Animal Ethics Committee (AEC) of University of Western Santa Catarina (protocol number 57/2018).

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