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Presence of *Cryptosporidium* spp. in calves from dairy herds in Northern Antioquia, Colombia

[*Ocorrência de Cryptosporidium* spp. *em bezerros de rebanhos leiteiros do norte de Antioquia, Colômbia*]

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ABSTRACT

Cryptosporidium spp. are important enteropathogen protozoan parasites that infect humans and other animals throughout the world. Cryptosporidiosis in cattle industry leads to considerable economic losses due to diarrhea, dehydration, growth retardation, weight loss, and possibly death, however, data on the occurrence of *Cryptosporidium* spp. in cattle in Colombia are limited. Therefore, this study aimed to investigate the occurrence and possible factors associated to the excretion of *Cryptosporidium* spp. oocyst in pre-weaned calves from dairy farms in Northern Antioquia, Colombia. In addition, Sheather's sugar floatation (SSF), and Modified Ziehl-Neelsen staining (MZN) methods were compared. A total of 41 fecal samples were collected from calves between 1 and 92 days of age of which 23 were positive (56.1%). Crossbreed calves were nine times less susceptible than purebred dairy cattle to excretion of *Cryptosporidium* spp. oocysts (OR=0.10). MZN was the best technique for the detection of oocysts in fecal samples, however, the mean number of days to detect cryptosporidial oocysts was lower for the SSF method. *Cryptosporidium* is widespread among calves under 2 months of age in dairy herds of Northern Antioquia, although further investigations considering a greater number of farms and animals are necessary.

Keywords: Antioquia, feces, coprological techniques, protozoa, risk factors, ziehl-neelsen

RESUMO

Cryptosporidium *spp. são importantes protozoários parasitas enteropatógenos que infectam humanos e outros animais em todo o mundo. A criptosporidiose na pecuária leiteira leva a perdas econômicas consideráveis devido à diarreia, à desidratação, ao retardo de crescimento, à perda de peso e possivelmente à morte, no entanto dados sobre a ocorrência de criptosporidiose em bovinos na Colômbia são limitados. Portanto, este estudo tem como objetivo investigar a ocorrência e possíveis fatores associados à excreção de oocistos de* Cryptosporidium *spp. em bezerros pré-desmamados de fazendas leiteiras no norte de Antioquia, Colômbia. Além disso, os métodos de flutuação de açúcar de Sheather (SSF) e de coloração de Ziehl-Neelsen modificado (MZN) foram comparados. Foram coletadas 41 amostras fecais de bezerros entre um e 92 dias de idade, das quais 23 foram positivas (56,1%). Bezerros mestiços foram nove vezes menos suscetíveis que bovinos leiteiros puros à excreção de oocistos de* Cryptosporidium *spp. (OR=0,10). MZN foi a melhor técnica para a detecção de oocistos em amostras fecais, entretanto o número médio de dias para detecção de oocistos foi menor para o método SSF.* Cryptosporidium *é amplamente difundido entre bezerros com menos de dois meses de idade em rebanhos leiteiros do norte de Antioquia, embora sejam necessárias mais investigações, considerando-se um maior número de fazendas e animais.*

Palavras-chave: Antioquia, fezes, técnicas coprológicas, protozoários, fatores de risco, Ziehl-Neelsen

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INTRODUCTION

Cryptosporidium spp. are important protozoan parasites that infect a wide variety of vertebrates including humans and farm animals (Feng *et al*., 2018). Bovine cryptosporidiosis is caused by *Cryptosporidium parvum, Cryptosporidium bovis, Cryptosporidium ryanae* and *Cryptosporidium andersoni*, but only *C. parvum* is associated with clinical disease in neonatal calves (Thomson *et al*., 2017). The latter is widely endemic and is considered to be the major zoonotic reservoir for humans (Ryan *et al*., 2021).

A diagnosis of cryptosporidiosis is based on the identification of *Cryptosporidium* spp. oocysts in the fecal sample by conventional, immunodiagnostic, and molecular methods. Several conventional techniques such as formal ether concentration-sedimentation followed by floatation on Sheather's solution (SSF), and unconcentrated stools by microscopy of smears stained with either modified Ziehl–Neelsen (MZN) are routinely used for the diagnosis of cryptosporidiosis (Pena *et al*., 1997; Chalmers *et al*., 2011).

Cryptosporidiosis is a worldwide infection in dairy calves. The presence of *Cryptosporidium* spp. in calves' feces has been studied in many countries, with values ranging from 50.5 to 96% (Trotz-Williams *et al*., 2008; Silverlås *et al*., 2009; Tiranti *et al*., 2011; Smith *et al*., 2014; Delafosse *et al*., 2015; Al Mawly *et al*., 2015). Risk factors for cryptosporidiosis in calves include young age (Brook *et al*., 2008) and environmental factors like housing (Kvác *et al*., 2006).

In Colombia (South America), there are a lack of knowledge about the epidemiology of *Cryptosporidium* in dairy calves. *C. parvum* has been reported in the major dairy regions of Colombia's provinces. In central Cundinamarca, Avendaño (2010) reported a prevalence of 22% (33/151) with MZN, and Pardo and Oliver (2012) reported a 38.6% (51/132) of prevalence with ELISA. In north Cundiboyacencian dairy herds a 7% (14/190) of prevalence with Heine stain was reported by (Montaño and Avendaño, 2012). Prevalence in the province of Caldas was 7.2% (11/80) with PCR (Ocampo *et al*., 2012). In Antioquia, the presence of *C. parvum* has been reported by Chaparro *et al*., (2014) who evaluated a cohort of 60 calves during the first month of life and found prevalence rates of 89.5% (51/57).

In general, data regarding the epidemiology of Cryptosporidiosis in dairy cattle in the province of Antioquia (Colombia) are still limited and only explored in the last 10 years, therefore, the aim of this study was to investigate the occurrence and potential risk factors associated to the excretion of *Cryptosporidium* spp. oocyst in pre-weaned calves from two dairy farms in Northern Antioquia, Colombia. In addition, Sheather's sugar floatation (SSF), and Modified Ziehl-Neelsen staining (MZN) methods were compared.

MATERIALS AND METHODS

All fecal samples used in this study were collected from cattle by veterinary surgeons with the permission of farm owners. The Bioethics Committee for Animal Investigation (CIBA) of the Corporación Universitaria Remington, Acta 08-2022, approved the procedures performed on the animals.

A total of 41 (35 females, 6 males) calves (*Bos taurus*) from two dairy herds located in Northern Antioquia-Colombia [San Pedro (6° 27′ 0″ N, 75° 33′ 0″ W); Entrerríos (6°33'55.44" N - 75°31'0.84" W)] were selected for the study. Fecal samples were collected from calves between 1 and 92 days of age by rectal extraction or by picking up fresh feces from the pen. The samples were processed a few hours after collection.

For the detection of *Cryptosporidium* oocysts in fecal samples, two techniques were used: 1) Sheather's sugar floatation method (SSF), and 2) Modified Ziehl-Neelsen staining technique (MZN). For the SSF technique, two grams of feces was taken and mixed in 10 ml of 10% formalin, and the suspension passed through a sieve (425 µm aperture size). To the filtrate was added 3 ml diethyl ether and the solution was shaken vigorously for 30 s and centrifuged at 500 x g for 3 min. The fluid and the fatty plug were discarded, and the pellet retained. To the sediment was added 10 ml of Sheather's sugar solution of 1.20 specific gravity; centrifugation was done at 500 × g for 3 min. *Cryptosporidium*

oocysts were observed under magnification (40x) for observation of internal morphology (Ferreira *et al*., 1962; Pena *et al*., 1997). The oocysts appeared as round or oval, refractile bodies with a thin cytoplasmic membrane and finely as pink granular cytoplasm (Figure 1A).

For the modified Ziehl-Neelsen staining (MZN), the protocol established by Casemore *et al*., (1984) was considered and some modifications were made. A smear of fecal material was made on each of the samples, dried at room temperature. Fixation and staining were done as follows: 1) fixation with methanol for 2 min (drying at room temperature), 2) staining with carbol-fuchsin for 5 min, (rinsing with tap water), 3) decolorization with alcohol acid 30 s (rinse with tap water), 4) counterstaining with methylene blue 1 min (rinse with tap water). The plate was allowed to dry at room temperature and was subsequently visualized under 100X objective with immersion oil. The oocysts appeared as round or oval, stained pink color (Figure 1B).

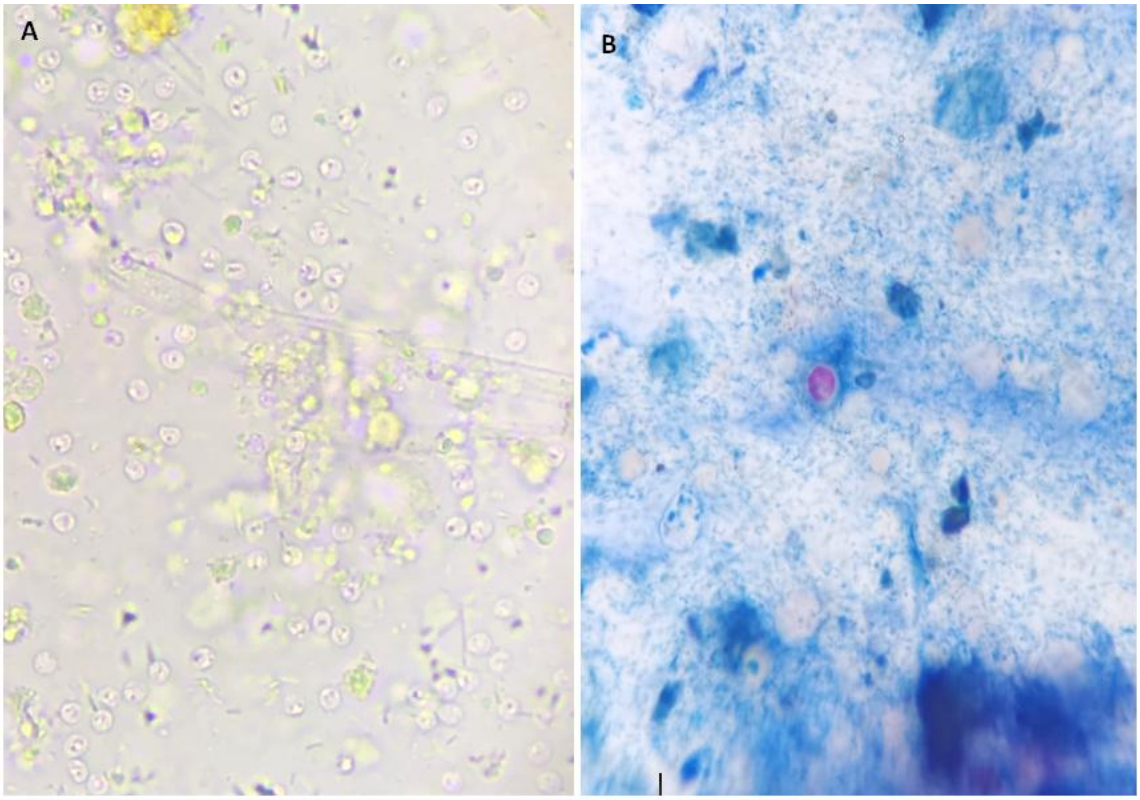

Figure 1. **A)** *Cryptosporidium* oocysts under sheather's sugar floatation method with magnification 400X. **B)** *Cryptosporidium* oocyst in modified Ziehl-Neelsen staining with magnification 1000X.

For Statistical analysis, farm and animal information was obtained throughout the application of a questionnaire to the farmers. The data generated from the interviews (independent variables) and the oocyst presence was the (dependent variable) were recorded and analyzed by R-language. A univariate analysis was performed using Fisher's exact test (F^2). The multivariate analysis was based on the logistic regressions in a model including the variables with $p \leq 0.25$ identified in the association test. An alpha error probability of less than 5% ($p < 0.05$) was considered statistically significant. The measure of agreement between the conducted tests was determined by kappa and McNemar's test. Interpretation of the κ value was based on the following categories of agreement: <0.2 (slight), 0.2–0.4 (fair), 0.4–0.6 (moderate), 0.6–0.8 (substantial), >0.8 (excellent) (Munoz and Bangdiwala, 1997).

RESULTS

A sample was considered positive for *Cryptosporidium* if an oocyst was detected through the SSF or MZN techniques **(**Figure 1A, and 1B**)**. Overall prevalence of *Cryptosporidium* spp. oocysts was 56.1% (23/41), with specific rates of 47% (8/17) at San Pedro, and 62.5% (15/24) at Entrerríos **(**Table 1**)**. In the F^2 univariate analysis, age results exhibited values in which calves >2 mo were less prone to likely to be positive to *Cryptosporidium* when compared to younger animals, however these differences were not statistically significant ($p > 0.05$; Table 1). In relation to breed, the multivariate analysis verified that crossbred (Jersey x Holstein, Holstein x Gyr) animals were nine times less susceptible than purebred dairy cattle to acquiring infection (OR = 0.10; Table 2). In addition, statistical differences were not found in farm location and fecal consistency. The mean number of days to detection of cryptosporidial oocysts was 30 for the SSF method and 41.3 for the MZN method.

Varying numbers of samples were positive by each of the diagnostic *Cryptosporidium* technique **(**Table 3**).** Of the 43 samples, 10 were positive by SSF method, and 20 samples were positive by MZN. There was 56% overall agreement between the SSF and MZN methods of oocyst detection with a kappa statistic of 0.11. The McNemar test indicated the proportion of tests determined positive by the two detection methods was not equal ($P<0.05$) showed that MZN was the most effective technique for the detection of oocysts.

Table 1. Univariate analysis of risk factor for *Cryptosporidium* infection in calves

Factor	No. of samples	Prevalence of *Cryptosporidium*		*p*-value[1]
		No. Positives animals	% (95% CI)	
Farm location				0.3583
San Pedro	17	8	47 (22.9-72.2)	
Entrerríos	24	15	62.5 (40.5-81.2)	
Age				0.0643*
< 1 month	15	10	66.6 (38.4-88.2)	
1-2 months	7	6	85.7 (42.1-99.6)	
> 2 months	19	7	36.8 (16.3-61.6)	
Breed				0.0313**
Holstein	34	22	64.7 (46.5-80.2)	
Other breeds	7	1	14.3 (0.04-57.9)	
Fecal consistency				0.3335
Soft	25	16	64 (42.5-82.0)	
Watery	16	7	43.7 (19.7-70.1)	
Overall prevalence	41	23	56.1 (39.7-71.5)	

[1]Fisher-exact test

Table 2. Multivariate analysis of risk factor for *Cryptosporidium* infection in calves

Factor	Odds ratio (OR)	95% CI* OR	*p*-value[1]
Age			0.2378
< 1 month	1		
1-2 months	0.22	0.04-1.05	
> 2 months	4.11	0.06-63.34	
Breed			0.0201
Holstein	1		
Crossbreed	0.10	0.01-0.99	

[1]Chi-square test

Table 3. A 2 x 2 contingency table for overall comparisons of Sheather's sugar floatation method (SSF) with modified Zielh-Neelsen technique (MZN) for detection of *Cryptosporidium* spp.

SSF	MZN		
	+	-	Total
+	6	4	10
-	14	19	33
	20	23	43

Per cent agreement 56%; kappa statistic 0.11 (SE±0.13); P<0.05

DISCUSSION

Cryptosporidium parvum is a zoonotic protozoan enteric parasite in bovine neonates. It causes chronic diarrhea leading to poor body weight, loss of yield and as an important cause of neonatal diarrhea and economic losses for the dairy industry. To the author's knowledge, this is the first report of the presence of *Cryptosporidium* spp. through two diagnostic techniques in dairy calves from the Northern region of Antioquia.

Overall prevalence of this study (56.1%; 23/41) was highest in contrast with the global prevalence (21.9%; 7755/42,890) of Cryptosporidiosis in dairy calves (Chen *et al*., 2023). Likewise, high presence of *C. parvum* was found compared with previous studies in South America such as Argentina (Lombardelli *et al*., 2019), Uruguay (Caffarena *et al*., 2021) and northeastern Brazil (Conceição *et al*., 2021).

More recent studies carried out on calves in Colombia indicated less prevalence of *C. parvum.* From 74 dairy cattle farms located in four departments of central Colombia (including 2 farms in Antioquia), *Cryptosporidium* prevalence was found to be 26.6% (115/432). Oocyst shedding was recorded in calves aged 3-day-old onwards, although the infection rate peaked at 8–14 days (40.7%) (Avendaño *et al*., 2018). In addition, our results indicated that dairy calves less than two months old are at more risk of a positive result compared with older calves. Age-related infection dependence of *Cryptosporidium* infection of calves has been recognized as one of the main risk factors (Santín *et al*., 2004; Kvác *et al*., 2006; Tiranti *et al*., 2011; Smith *et al*., 2014; Delafosse *et al*., 2015; Avendaño *et al*., 2018), however, this variable did not persist in the multivariable analysis.

In this study, purebreds were more susceptible to excreting *Cryptosporidium* oocysts when compared to crossbreed. Nevertheless, these results must be analyzed with caution, since many of the sampled animals belonged to the Holstein breed (79%), therefore, it would be inappropriate to compare whether there is a breed predisposition to the presence of *Cryptosporidium*. However, some studies consider that purebreds are more vulnerable to infection when compared to crossbreeds (Imre *et al*., 2015; Brainard *et al*., 2020). More studies with a larger number of samples from different breeds are necessary.

Diagnosis of *Cryptosporidium* spp. include different laboratories approaches. Although the detection and diagnosis of Cryptosporidiosis has many challenges including performance, cost, and time, microscopy techniques can boost reliance in the diagnosis (Abdou *et al*., 2022). The SSF method has shown good performance for *Cryptosporidium* spp. diagnosis in dairy calves (Rekha *et al*., 2016), small ruminants (Pavlović *et al*., 2020), even in humans (Manthalkar *et al*., 2017). Nevertheless, Danišová *et al*. (2016) considered PCR the gold standard reference test because PCR has high accuracy, however, a full screening of *Cryptosporidium* infection in fecal samples include the use of composite reference standard as a gold standard (Abdou *et al*., 2022). Although the SSF method requires reduced labor time when compared to MZN technique, the kappa agreement between MZN and SSF in this study was low. It could be explained because there is not a standard recommendation for the recovery of *Cryptosporidium* oocysts from samples with low infection (Weber *et al*., 1991). However, future studies may contemplate repeated examination of more than one fecal sample on three consecutive days enhancing the detection of *Cryptosporidium* oocysts. SSF technique is a rapid method of oocysts detection. Oocysts are easily identified and present as pink spherical bodies inside. This technique could be

applied with other molecular and immunological assays for future studies in this dairy population.

Limitations of this study include the use of a convenience sample (selection bias). Nevertheless, due the lack of information around *C. parvum* infection in calves from the dairy region of Northern of Antioquia, our exploratory study demonstrates the first effort for determine the presence of Cryptosporidiosis in two municipalities in this dairy region. Further research is needed to understand the epidemiology of Cryptosporidiosis in calves in the northern of Antioquia, linking the role of *C. parvum* with animal and human health.

CONCLUSION

In conclusion, the high rate of presence of *Cryptosporidium* in two farms in northern Antioquia demonstrates the persistence of this protozoan in the country's dairy industries. This study identified that purebreds are more susceptible to crossbreeds when compared, however, studies involving a larger number of farms, and other potential risk factors are needed to determine appropriate measures that farmers and veterinary practitioners should take to reduce the prevalence of *Cryptosporidium*.

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