

## Risk factors associated with the occurrence of *Cryptosporidium parvum* infection in calves

[Fatores de risco associados à ocorrência de infecção por *Cryptosporidium parvum* em bezerros]

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### ABSTRACT

*Cryptosporidium parvum* oocysts were detected in feces of dairy calves raised in Rio de Janeiro State and the risk factors involved in the infection were determined. A hundred calves aging up to 12-month-old from 13 dairy farms were sampled. Polymerase chain reaction was used to detect the presence of oocysts. The zoonotic *C. parvum* species was detected in 45% animals. Statistical risk factors analyses revealed an association between infection and animals raised in technical systems such as the use of milking equipment, milking cooler, and water trough ( $P < 0.05$ ).

Keywords: dairy calf, criptosporidiosis, protozoan, risk factors

### RESUMO

Detectaram-se oocistos de *Cryptosporidium parvum* em fezes de bezerros leiteiros no estado do Rio de Janeiro e analisaram-se os fatores de risco envolvidos na infecção dos animais. Cem bezerros com idades de 0 a 12 meses, provenientes de 13 propriedades rurais, foram amostrados, e suas fezes examinadas pela reação em cadeia da polimerase para a detecção dos oocistos. A espécie zoonótica *C. parvum* foi detectada em 45% dos animais. As análises estatísticas dos fatores de risco revelaram haver associação entre infecção e animais criados em propriedades tecnificadas, que usam ordenha mecanizada, resfriamento de leite e fazendas que continham reservatórios de água à disposição dos animais ( $P < 0,05$ ).

Palavras-chave: bovino, criptosporidiose, protozoário, fatores de risco

### INTRODUCTION

Protozoan from the genus *Cryptosporidium* causes diarrhea in a wide range of vertebrates including humans (Current et al., 1983). Although they have been reported in people from three days to 95 years old, the highest infection prevalence has been recognized in both children and young animals, and they are reported as a major cause of self-limiting diarrhea in immunocompetent people (Fayer et al., 1990; Chen et al., 2002). *C. parvum* is a particularly

serious health threat to immunodeficient individuals, having no effective treatments for the disease (Kuczynska and Shelton 1999). *Cryptosporidium* oocysts have been reported to cause several water-borne and food-borne outbreaks worldwide (Smith and Rose, 1998; Fayer et al., 2000), and are resistant to disinfectants commonly used in drinking water treatment, consequently, the detection of oocysts is important to the water industry (Xiao et al., 2000; Liao et al., 2001). Most human infections are due to *C. parvum* (Ramirez et al., 2004), and fecal food and drink contamination as well as

person-to-person contact with oocysts are the most common methods of enteric protozoan transmission (Laubach et al., 2004).

The prevalence of *C. parvum* infection in animals is high (Villacorta et al., 1991; Ederli et al., 2004; Almeida et al., 2008). Dairy and beef calves are generally considered as presenting the highest risk because of their numbers, distribution, high infection occurrence, and high oocyst excretion levels (Kuczynska and Shelton, 1999), besides be exposed to contamination environment sources, such as soil and water.

Taking into account the high prevalence of *C. parvum* infection in calves and humans, and the lack of researches involving risk factors for this protozoan in Brazil, the objective of this work was not only to discuss the occurrence of this parasite, but also to detect the risk factors for contamination in different breeding systems.

## MATERIAL AND METHODS

The study was carried out in 13 farms located at the North of Rio de Janeiro State, Brazil. Faecal samples from 100 calves (50 males and 50 females), aging up to 12-months-old, were collected, regardless symptoms of diarrhea.

Data were collected from a questionnaire on factors hypothesized to be associated with the risk of *C. parvum* infection in calves, such as farm size, water source, animal gender, flooring type, management, and different breeding systems.

To clean *Cryptosporidium* oocysts, 15g of feces were suspended (vortex) in 35mL of deionized water, then filtered through a 325-mesh wire screen. After centrifugation at 2500g for 10min, the pellet was resuspended in deionized water to a final volume of 25mL and an additional 25mL sucrose solution (1.1g/cm<sup>3</sup>) was added. The material was thoroughly mixed, and centrifuged at 250g for 20min. Four milliliters of supernatant were aspirated from the top, pipetted into a conical tube, then deionized water was added to the solution to reach a final volume of 15mL. The suspension was centrifuged at 2500g for 10min, and the pellet was resuspended in 500μL deionized water, being the final solution stored at -20°C.

For the DNA extraction, 50μL of the concentrated material were placed in 1.5mL microcentrifuge tube containing 0.5mL of DNA extraction buffer (Tris 50mM, NaCl 100mM, EDTA 0,05mM). Proteinase K and SDS were added to final concentrations of 1.7mg/mL and 2.5%, respectively, and they were added to the mixture. The samples were stored at 55°C overnight in stirred water bath. After these steps, 700μL TE-saturated phenol-chloroform were placed into each tube, and after manual water agitation at room temperature, samples were centrifuged at 12000g for 10 min. The upper aqueous phase was collected and transferred to another 1.5mL microcentrifuge tube, and DNA was precipitated with sodium acetate and isopropanol. The tubes were stored on ice for 15 minutes, then centrifuged at 12000g during 20min. The supernatant was discarded, 1mL cold ethanol 70% was added, and after inverting the tubes, the samples were centrifuged at 12000g for 5min. The tubes were inverted on a paper wipe for a few minutes and dried completely at 38°C. Finally, the DNA extracted was suspended in 50μL ultra pure water.

Polymerase chain reaction amplification was performed in 20μL total volume and included 2μL extracted DNA. For the 18S rRNA reaction, the following concentrations of the various reagents were used: 1xPCR buffer, 3mM MgCl<sub>2</sub>, 0,2mM each dNTP, 1.5 units Taq DNA polymerase, and 50pmol each forward and reverse primers. For the Nested PCR (secondary amplification), the following concentrations of reagents were used: 1xPCR buffer, 1.5mM MgCl<sub>2</sub>, 0.2mM each dNTP, 1.5 units Taq polymerase, and 50pmol each forward and reverse primers. The primers sequences used in the reactions were: CryptoF: 5' TTC TAG AGC TAA TAC ATG CG 3' and CryptoR: 5' CCC TAA TCC TTC GAA ACA GGA 3' (primary amplification) and AL1598: 5' AAG GAG TAA GGA ACA ACC TCC A 3' and AL3032: 5' GGA AGG GTT GTA TTT ATT AGA TAA AG 3' (Nested PCR). For primary amplification, reaction mixtures were denatured at 94°C for 3min, then subjected to 35 cycles of 45s at 94°C, 45s at 59°C, and 60s at 72°C, and a final 7min extension at 72°C. The Nested PCR was performed at 94°C for 1min, then subjected to 40 cycles of 30s at 94°C, 90s at 58°C, and 2min at 72°C, and a final 7min extension at 75°C. The PCR products were analyzed by electrophoresis

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on horizontal agarose gels (2%) in TAE buffer (40mM Tris acetate, 2mM Na<sub>2</sub>EDTA), stained with ethidium bromide and visualized under a UV transilluminator.

Comparisons of infection occurrence rates among the groups were made by the Chi-square statistic test or the Fisher Exact Test with Yates correction and 95% confidence interval. Among groups, differences were tested for significance at the 5% level of probability (P<0.05).

**RESULTS**

The number of samples collected from each farm ranged from one to 11, with an average of five samples per farm. DNA fragments of the expected size were obtained in 45 samples (45%) from 11 out of the 13 (84.6%) examined farms (Table 1).

Table 1. Results of polymerase chain reaction for *Cryptosporidium parvum* in dairy calves according to the farms

Farm	Samples		%
	Number of calves examined	Positive	
1	7		0
2	1	1	100
3	11	4	36.4
4	7	4	57.1
5	7		0
6	9	3	33.3
7	6	4	66.7
8	8	7	87.5
9	9	3	33.3
1	7	1	14.3
11	8	4	50
12	9	9	100
13	11	5	45.5
Total	100	45	45.0

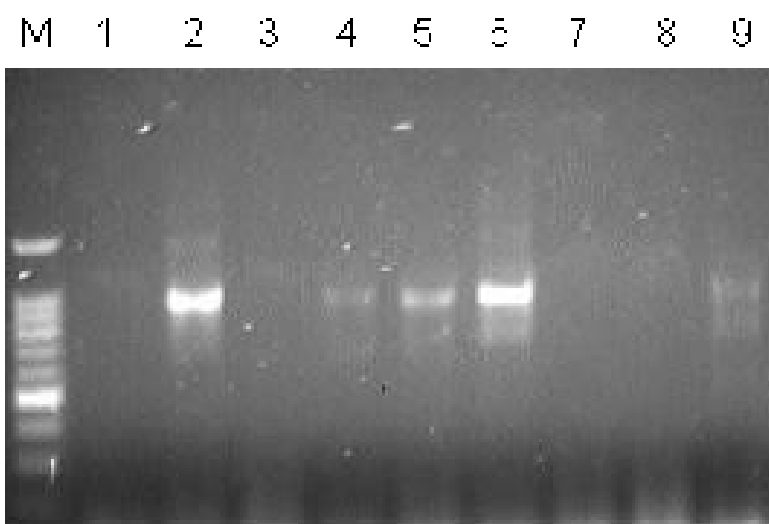


Figure 1. Detection of *Cryptosporidium parvum* in feces by polymerase chain reaction. M: 100bp ladder of molecular weight marker. Lane 1; negative control. Lanes 2, 4, 5, 6, and 9; positive samples. Lanes 3, 7, and 8; negative samples.

Several factors were not associated with the shedding of *C. parvum*. The farm size (up to 100 and more than 100 hectares) did not have any significant effect on the risk of infection by *C. parvum* (P=0.06; Rr=0.70: 0.49-0.99); as well as the water source, whether from waterfalls or lakes or rivers (P=0.27; Rr=1.13: 1.37-0.84); the flooring type, whether made of wood or concrete (P=0.23; Rr=1.22: 0.92-1.61); and hygiene conditions, being considered good or bad, according to the observations (P=0.33; Rr=0.75: 0.46-1.21).

On the other hand, some other factors were significantly associated with the risk of *C. parvum* infection. The use of milking equipment was one of the considered risk factors, and results showed significant differences in the prevalence of cryptosporidiosis in farms having or not milking equipment. Other variables studied, such as the presence of a milking cooler and water trough were both shown as having significant influence for the *C. parvum* infection in the studied farms. The statistic analyses of these variables are showed in Table 2.

Table 2. Relative risk of *Cryptosporidium parvum* infection in calves

Variable		Positive	Negative	Fisher exact test (CI 95%)
Milking	Hand	16	40	P<0.0001
	mechanized	26	09	Rr= 0.46 0.31<Rr<0.70
Use of milking cooler	Yes	25	09	P<0.0001
	No	13	33	Rr= 3.07 1.16<Rr<5.72
Presence of trough	Yes	33	19	P= 0.0001
	No	09	30	Rr= 2.02 1.37<Rr<2.98

## DISCUSSION

In recent years, concern has increased regarding the potential public health hazard of water supplies contamination by *C. parvum*. Dairy herds are one of the potential sources of this parasite (Angus, 1991). The high percentage of positive samples (45% of analyzed animals and 84.6% of visited farms) showed the high occurrence of the parasite and possible environmental contamination due to the infected animals. Results reported in different countries show values ranging from 13 to 100% of properties contamination and 0.9 to 61% of parasite-infected calves, with high prevalence in Brazil (Wade et al., 2000; Fayer et al., 2006; Park et al., 2006; Castro-Hermida et al., 2007; Almeida et al., 2009).

In a recent study done in São Paulo, Féres et al. (2009) observed that 38.1% (8/21) of properties that raised lambs had positive animals for *Cryptosporidium*, with an incidence of 6.7% (31/460) in animals, having no significant difference between the breeding system and number of positive samples. Féres et al. (2009)

identified a sample of *C. parvum* 18S rRNA type A, what is most common in ruminants infections, and also a strain of *C. parvum* 18S rRNA type B, all with zoonotic potential.

Studies show that the effectiveness of *Cryptosporidium* oocysts recovery methods depends on the sample nature and the used methodology for oocysts concentration (Machado et al., 2009), which may explain the wide variation of results related to protozoan occurrence.

The presence of water trough was significantly associated with the relative risk of infection by *C. parvum*. This association can be explained by the fact that the water trough is probably potential reservoirs of *Cryptosporidium*, since the oocysts can survive in water for a long period of time (Simmons III et al., 2001; Luna et al., 2002) and probably there is constant fecal contamination of that water by animals and even by humans. Thus, hygiene methods to clean and disinfect water troughs should be taken into account as important preventive strategies.

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Machado et al. (2009) analyzed water from collection sites in Pernambuco and found *Cryptosporidium* oocysts in both raw and treated water, and reported the presence of a riverside location cattle pen close to a water collection site.

The raising of calves under technical systems was considered a factor that was statistically associated with the shedding of *C. parvum*. Interestingly, farms having milking equipments and milking coolers were more likely to have calves infected with the parasite than farms that did not have the equipments, in accordance with observations done by Feitosa et al. (2004) and Almeida et al. (2009), in Brazil as well. This result is somewhat difficult to explain, but it might be related to the higher levels of stress and physiologic demands suffered by adult animals, being submitted to commercial breeding systems, compared to calves bred under family raising systems. Then, probably the juveniles are being contaminated by the high number of older stressed calves.

It was interesting to observe that no association was found between different hygiene conditions and the likelihood of infection with *C. parvum*, what is in accordance with the findings by Maldonado-Camargo et al. (1998) in Mexico and Maddox-Hyttel et al. (2006) in Denmark. Those data are worrying since they show how difficult it is to prevent parasite dissemination.

Similarly to findings by Tarazona et al. (1998) and Lallo and Bondan (2006), who detected *Cryptosporidium* in mice and dogs, respectively, there was no relation between *Cryptosporidium* infection and the animals gender. Also, farm sizes and water sources had no evident influence on the occurrence of infection by the protozoan.

### **CONCLUSIONS**

There is high prevalence of protozoan infections by *Cryptosporidium* genus in calves raised in Campos dos Goytacazes, a county in Rio de Janeiro State, indicating that cryptosporidiosis occurs endemically in flocks. Calves raised in technically operating farms using mechanical milking, milk cooling, and water tanks showed higher infection risk by the parasite.

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