Cryptosporidium parvum in diarrheic calves detected by microscopy and identified by immunochromatographic and molecular methods

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ABSTRACT

Cryptosporidium is an important protozoan parasite that causes diarrhea in neonates and young bovines. The objective of the present study was to determine the frequency of *Cryptosporidium* infection in animals of dairy farms of the Metropolitan Region (Santiago), Chile. Fecal samples of 205 newborn calves with diarrhea were studied and used for comparing the efficiency of two microscopic staining methods for diagnosis of the parasite, the auramine (AU) and a modified Ziehl-Neelsen (ZN) procedure. Out of the 205 fecal samples, we detected oocysts in 115 (56.1%) with AU and 102 (49.8%) with ZN. Comparison of results obtained with the two microscopic techniques showed significant difference ($p < 0.05$), AU being more sensitive. On the other hand, concordance between the two methods was almost perfect (kappa value of 0.83). The results with these two operator dependent methods were confirmed using an operator independent immunochromatographic (IC) method. The IC method also enabled us to determine the identity of the parasite species as that of *Cryptosporidium parvum*. Identification of the parasite species was further corroborated by performing a *Cryptosporidium* species-specific polymerase chain reaction (PCR) test on few samples taken at random. Overall, the results showed a high number of infected animals suggesting the parasite *C. parvum* as a major parasitic disease agent of neonatal calves with diarrhea in dairy farms of the Metropolitan Region (Santiago) of Chile.

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1. Introduction

Enteritis and diarrhea are leading causes of calves' death during their first weeks of life. A wide range of causative infectious agents may be involved in this pathology. In general, the main causes are bacterial infections, although viral and protozoan agents can also infect the animals concomitantly or exclusively within the 10–14 days of age. It has been reported that calves infected with the protozoan *Cryptosporidium* spp. are usually asymptomatic, and only stressed animals or individuals with a concomitant pathology show clinical manifestations of the disease named cryptosporidiosis (Blowey and Weaver, 2003). *Cryptosporidium* spp. affects the digestive tract of many vertebrates, including humans, farm animals (cattle, sheep, goats, horses, chickens and turkeys), pets (dogs and cats), laboratory animals (rats and mice) and wild birds, reptiles and fishes (Gómez-Couso et al., 2005). In most of them, *Cryptosporidium* spp. grow and multiply in the microvillus borders of the enteric epithelium cells. The parasite may additionally infect other tissues such as the respiratory and renal epithelia, especially in immuno-compromised humans (Mercado et al., 2007; Fayer and Xiao, 2007). When oocysts are ingested by a suitable host, the endogenous phase of the parasite life cycle begins by invasion of target cells. This biological process includes schizogony, gametogony, fertilization, and sporogony (Bowman, 1999; Fayer and Xiao, 2007). The sporulated oocyst is the only exogenous stage in the life cycle of the parasite, and being excreted with the feces of an infected host (Fayer and Xiao, 2007) is highly relevant for the diagnosis of *Cryptosporidium* infection. It is eliminated in great quantities with fecal material and is capable of survival for long periods in the environment (Bowman, 1999; Naciri et al., 1999; Neira, 2005; Fayer and Xiao, 2007). The parasite infects a wide range of hosts and has a cosmopolitan geographic distribution that includes the Antarctic continent (Fredes et al., 2007, 2008). *Cryptosporidium* infection is most frequent in calves less than one month of age, clinically characterized by varying degrees of diarrhea. In immuno-compromised humans it is a severe disease, difficult to treat and can be fatal if extreme dehydration occurs (Sunnotel et al., 2006). Presently, there is no specific anti-parasitic chemotherapy for the treatment of *Cryptosporidium* infections (Caccio and Pozio, 2006). Cryptosporidiosis in cattle is mainly caused by *C. parvum* (Sunnotel et al., 2006). Other species involved are: *C. andersoni*, *C. bovis* and *C. ryanae* (*C. parvum* deer-like genotype) (Fayer, 2010). The disease in calves has been studied in many countries, with prevalence ranging between 2.4 and 100% (Quílez et al., 1996; De La Fuente et al., 1999; Wade et al., 2000; Castro-Hermida et al., 2002a,b; Fayer and Xiao, 2007). In Chile, updated information on the prevalence of *Cryptosporidium* in dairy calves is almost non-existent. The last reported survey was performed by Gorman et al. (1989), who reported an infection rate of 23.2% in diarrheic calves less than one month old. No species identification of the parasites was performed at the time. We report here an updated rate of *Cryptosporidium* infection in cattle rearing farms from the Metropolitan Region of Chile. For this purpose, we used in a first step, two (operator dependent) microscopy methods, the auramine (AU) and the Ziehl-Neelsen acid-fast staining method (ZN) and compared the two methods for sensibility and agreement. We next used an immunochromatographic method (IC), which is operator independent, as a benchmark for the results obtained with AU and ZN and to aid in the identification of parasite. Finally, the identity of the parasite species was corroborated to be *C. parvum* by testing few samples, from which we obtained DNA, with a *Cryptosporidium* species-specific polymerase chain reaction (PCR) (Mercado et al., 2007).

2. Materials and methods

2.1. Sample size

We worked with fecal samples of 205 diarrheic calves. This sample size was used for comparison of two proportions using a theoretical sensitivity of 70% by ZN and 85% by the AU test, with 95% confidence and 95% power. The sample size was calculated so to detect in one field working year the required number of animals proportional to the total animal population living in the farms.

2.2. Study period and area

Samples were obtained from two different dairy cattle farms in the Metropolitan Region (Santiago), Chile, with the consent of the owners' farms. Fresh stool samples were collected from diarrheic calves which age ranged from four days to four weeks. The collection of fecal samples was initiated in August of 2007 and ended in April 2008, corresponding to the austral winter and spring seasons of 2007, and summer and autumn of 2008 respectively, period in which the calculated sample size was reached.

2.3. Sample collection and processing

The samples were obtained directly from the rectum of female animals. Samples were collected in two 50 mL plastic tubes containing formaldehyde to 10% (Weitz and Tassara, 1989) or ethanol to 70% (Mercado et al., 2007). Samples identified by collection date, calf number and birth date were temporarily stored in an ice container and taken to the Laboratory of Parasitology, Faculty of Veterinary and Animal Sciences, University of Chile. Processing consisted of filtering the samples through a filter (bronze metal net) and centrifugation at $1500 \times g$ for 15 min. For ZN and AU staining, the sediment of samples in formaldehyde was suspended in 500 μL of formaldehyde to 10%. Fifty microliters of the suspension was spread on a glass slide, air dried and processed accordingly. For IC identification of parasite we followed the instructions of the kit manufacturer (Cryptostrip, CORIS Bioconcept, Belgium).

DNA extraction was performed on 200 μL equivalent of fecal sample preserved in ethanol 70%. After centrifugation the sediment was resuspended in 200 μl of TE buffer (10 mM Tris–HCl, 1 mM EDTA pH 8.0) and boiled for 1 min. Proteinase K at the final concentration of 50 mg/mL was added and the suspension incubated overnight at 65 °C in the presence of 10% sodium dodecyl sulfate. The solution with the digested material was then sequentially

treated with phenol, phenol-chloroform-isoamyl alcohol, and chloroform-isoamyl alcohol as described for DNA extraction. The DNA in the aqueous phase was then precipitated with sodium acetate (3 M, pH 5.2) and ethanol, and after centrifugation, the pellet dissolved in 50 μL of water.

2.4. ZN and AU staining

Samples were stained and microscopically screened for *Cryptosporidium* oocysts using the ZN (Casemore, 1991) and AU (Fayer and Xiao, 2007) methods. No other enteropathogen was studied in the samples. A sample was considered positive for *Cryptosporidium* if at least one oocyst was detected upon direct microscopic (100×) exam, according to the criteria of oocyst optical properties, internal structure, size and shape, as described in Fayer and Xiao (2007).

2.5. Immunochromatographic test (IC) kit (Llorente et al., 2002)

A diagnostic immunochromatographic test (IC) kit (Cryptostrip, CORIS Bioconcept, Belgium) was used for identification of parasite in 22 randomly selected samples that were either positive (11) or negative (11) by the ZN or AU microscopic methods. The sample size (n = 11) was determined for the probability of 0.95 of finding at least one positive sample in a population of 120 diarrheic calves with and without *Cryptosporidium* infections detected by microscopical methods, with a prevalence of 25% (Gorman et al., 1989) and a confidence limit of 95% (Thrusfield, 2005).

2.6. Polymerase chain reaction (PCR) for the identification of Cryptosporidium species

We used a species-specific PCR assay (Mercado et al., 2007) to determine the species of *Cryptosporidium* in the samples. *C. parvum* isolate Moredun (Okhuysen et al., 2002) (Fig. 2, lane 5 and 11, Cp) and *C. hominis* isolate TU502 (Widmer et al., 2000) (Fig. 2, lane 6 and 12, Ch) DNA's were used as PCR controls. In the species-specific PCR assay, a *C. hominis* DNA fragment of ∼400 bp is amplified with the primer Lib13SF02 (5′-TTTTTTCATTAGCTCGCTTC-3′) combined with the *C. hominis* specific anti-sense primer Lib13SRT-1 (5′-ATTTATTAATTTATCTCTTACTT-3′). Conversely, a *C. parvum* DNA fragment of about the same size is amplified when the primer Lib13SF02 is used with the *C. parvum* specific anti-sense primer Lib13SRT-2 (5′ATTTATTAATTTATCTCTTCG-3′). Amplifications were carried out in a PCR mixture of 10 μL containing 0.25 mmol/L of each dNTP, 300 pmol/L of each olignucleotide, and 1 unit of Taq DNA polymerase (HotMaster, 5 PRIME, Inc., Gaithersburg, MD, USA). Temperature cycling was performed on a GeneAmp PCR System (ABI, Foster City, CA, USA) with initial denaturation performed at 95 °C for 5 min, then 40 cycles at 95 °C for 30 s, 52 °C for 30 s, and 68 °C for 30 s. The mixture was then cooled to 4 °C prior to analysis by agarose gel electrophoresis. We obtained DNA from three fecal samples that were simultaneously

Table 1
Number of fecal samples positive for *Cryptosporidium* oocysts within the 205 samples of diarrheic calves, using the Ziehl-Neelsen (ZN) or Aureamine (AU) method.

Method	Number of animals with oocysts		Total n
	n	%	
ZN	25(A)[a] + 77(B)[a]	49.7	102
AU	34(A) + 81(B)	56.1	115

[a] Dairy farms A (A) and B (B).

ZN, AU and IC positive for *Cryptosporidium* and performed the identification assay.

2.7. Statistical analysis

The results were analyzed using the McNemar test for correlated or dependent proportions (Remington and Schork, 1970). In addition, the kappa coefficient was used to determine levels of agreement between ZN and AU staining tests (Fleiss, 1981).

3. Results

We detected *Cryptosporidium* oocysts in 49.8% and 56.1% of the 205 diarrheic calves studied during 2007–2008, by the ZN and AU staining methods respectively. The results obtained from the animals of the two surveyed dairy farm are presented in Table 1. Differences in detection sensitivity between AU and ZN were significant ($p < 0.05$) being AU more sensitive than ZN for detecting oocysts. However, the level of agreement between both tests was almost perfect (kappa value = 0.83).

Fig. 1 shows monthly distributions of animals infected with *Cryptosporidium* sp. in both dairy farms as diagnosed by ZN or AU. Occurrence of the parasite in the surveyed animals was observed during the entire period of the survey.

The frequency of *Cryptosporidium* infection by age was also recorded. The highest intensity of infection was observed in animals between 7 and 14 days old (63%; data not shown).

To confirm the results obtained with the AU and ZN staining methods, and to aid in the identification of the *Cryptosporidium* species, an immunochromatographic (IC) test was applied to a total of 22 samples, 11 oocyst-positive with both ZN and AU microscopy methods and 11 oocyst-negative samples by the same criteria. *C. parvum* was the species of *Cryptosporidium* identified with IC in 10 out of the 11 samples oocyst-positive by ZN and AU, or 91%. No further parasite identification in the 11th sample was attempted. All eleven oocyst-negative samples were also negative by IC. Three samples that were parasite positive in all three assays were further tested with a species-specific PCR assay (Mercado et al., 2007). The *Cryptosporidium* species identified by the PCR method in the three samples was *C. parvum* (Fig. 2).

4. Discussion

The first report of *Cryptosporidium* spp. infection in cattle in Chile was published by Gorman et al. (1986). They
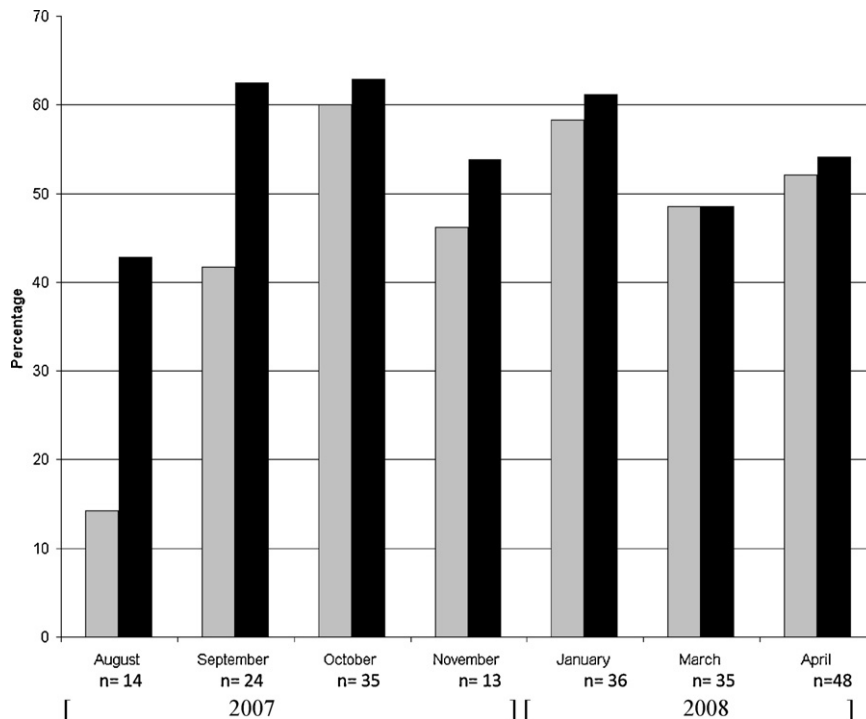
Fig. 1. Percent of diarrheic animals with *Cryptosporidium* oocysts as detected by Ziehl-Neelsen (ZN) (grey bars) or Aureamine (AU) (black bars) staining. Samples (total *n* = 205) are grouped for the month of the years 2007 and 2008 they were collected, as indicated.

found 12 out of 51 diarrheic calves (23.5%) with the para-site oocysts. None of 66 non-diarrheic calves in the study showed oocysts. In another study, the same group found an average rate of 12.8% of *Cryptosporidium* spp. infection

in animals from the Metropolitan Region (Santiago), Chile. When considering healthy animals the infection rate was 2.3% while in diarrheic animals the rate was 23% (Gorman et al., 1989). Campano (1997) using an immunofluorescent
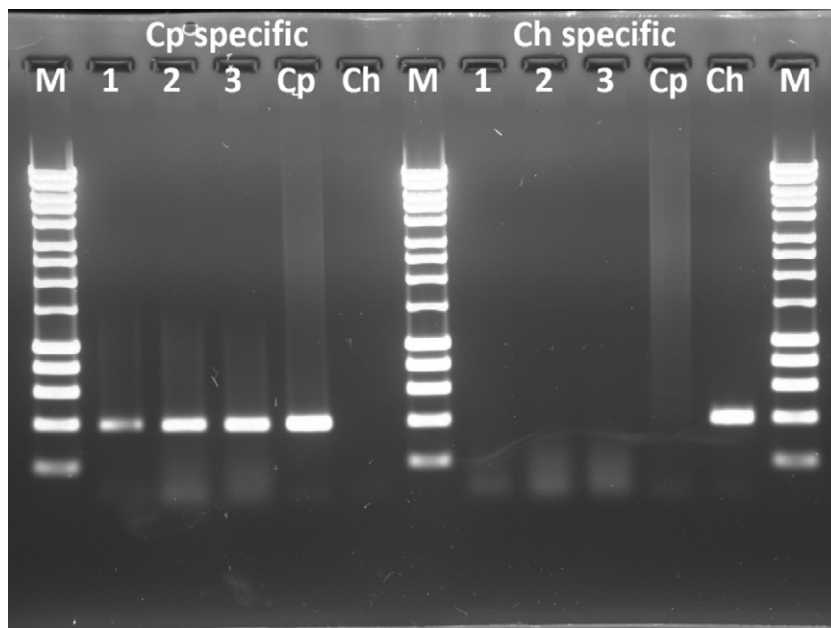

Fig. 2. Identification of *Cryptosporidium* species in DNA from fecal samples of three animals positive for the parasite by ZN, AU and IC with the *Cryptosporidium* species-specific Lib13 PCR assay (Mercado et al., 2007). M, DNA molecular marker (HyperLadder I, Bioline, Randolph, MA, USA, second band from bottom is 400 bp); 1, 2 and 3, DNA from the three selected animal samples; Ch, *C. hominis* DNA; Cp, *C. parvum* DNA.

diagnostic method, reported an infection rate by *Cryptosporidium* spp. of 30.6% of diarrheic calves in farms of a Southern Region of Chile. Our results revealed a higher frequency of infection by the parasite, more than twice the rate communicated by Gorman et al. (1986, 1989). Though restricted to only two sites, our sampling results suggest that cryptosporidiosis is highly endemic and that *Cryptosporidium* could be considered as a re-emergent agent of diarrheic disease in bovines at least in this region of Chile.

Intensive dairy cattle production, overcrowding, poor hygiene and inadequate management measures are risk factors for diarrheic diseases in farms. In the surveyed farms hygienic conditions and animal stock management were appropriate, with calves housed individually after delivery, and correct administration of colostrum and concentrated food. However, calves were housed in such a way that direct contact between the animals was possible. It has been reported that management of animals in which calves are housed with their mothers and without contact with other calves tends to decrease the prevalence of *Cryptosporidium* infections (Kvác et al., 2006).

Other important risk factors of cryptosporidiosis spreading in newborn ruminants are the method and frequency of pen cleaning and the floor type of the pen. Infection risk decreases when calves are housed individually in pens with a cement floor previously disinfected, and daily washing with water under pressure drag is used (Castro-Hermida et al., 2002a, 2006).

Studies have shown a direct association between age of animals and the risk of *Cryptosporidium* infection, most of them occurring between the first and fourth week of life (Castro-Hermida et al., 2002b). The parasite has not been found in animals younger than 4 days old (Sturdee et al., 2003; Kvác et al., 2006) and is only detected after 7 days of life (Del Coco et al., 2008). The highest reported infection rates are with calves between 7 and 14 (Kvác et al., 2006) or 8 and 21 days old (Del Coco et al., 2008). In the present study, the highest rate of infection we observed is in animals between 7 and 14 days old (63%).

We found AU more sensitive than ZN for the detection of *Cryptosporidium* sp. oocysts in bovine fecal samples ($p < 0.05$). This contrasts with previous reports using the same staining methods, one with 29 fecal samples and another with 81 samples. Both studies showed no significant differences between the results obtained with the two techniques ($p > 0.05$). However, the level of agreement of the results was just substantial (kappa value = 0.79) (De Quadros et al., 2006; Brook et al., 2008). The relatively small sample size in these two studies may have influenced the results. In the present study we found an almost perfect level of agreement between the results with the two methods (kappa value = 0.83).

The diagnostic efficiency of the staining methods is extremely operator dependent. The IC test is an alternative for a rapid, operator independent, diagnosis of cryptosporidiosis, although more expensive. The combined assays using AU, ZN and IC, did not resulted in an exact concordance of parasite detection. Nevertheless, our results suggest that the combination of AU and IC might give an acceptable sensibility level and confidence for *Cryptosporidium* detection in dairy cattle.

We demonstrated with the IC test that the oocysts observed with the two staining methods corresponded to *C. parvum*, and were not only artifacts or contaminating detritus. Furthermore, the species of the parasite in three fecal samples, which were parasite positive in all three tests, AU, ZN and IC, was confirmed to be *C. parvum* by using a specie-specific PCR method (Mercado et al., 2007). The identification of *Cryptosporidium* species using PCR assay, although not able to identify other known species apart from *C. hominis* or *C. parvum* enables us to further confirm the species infecting the calves. The three samples for species identification by PCR were randomly selected as a representation of samples collected between 2007 and 2008. This is the first clear identification of *C. parvum* infection in cattle in the Metropolitan Region in Chile using a PCR assay. *C. parvum* identification in Chilean bovine by PCR was also reported in the Valparaiso Region Neira-Otero et al. (2005) who found one infected out of 127 adult animals studied.

Our results show that *C. parvum* continues to be a highly enzootic disease agent at least in some locations of Chile. As a zoonotic pathogen, soil and water contamination by cattle infected with this parasite species, greatly contributes in the maintenance of both human and animal cryptosporidiosis. More studies are needed to determine an accurate epidemiological and enzootic profile of this parasitosis in the Country.

5. Conclusion

Our results suggest that, in the Metropolitan Region of Chile, *Cryptosporidium* is one of the leading cause of diarrhea in neonatal calves. We also obtained evidences, with the IC and the cryptosporidium species specific assays, that the oocysts present in the fecal samples of neonatal calves are those of *C. parvum*. Though no regular survey has been taken concerning cryptosporidiosis in Chile, this study suggests a significant increase in the frequency of infection, especially in calves, and therefore should be seen as a re-emerging infectious disease of cattle in this country. In our hands the AU test was more sensitive than ZN for the detection of *Cryptosporidium* oocysts in fecal samples ($p < 0.05$), with an almost perfect level of concordance of the results between the two tests. We also used the IC test for confirming AU and ZN methods for parasite detection and for parasite species identification. Our results suggest that a combination of AU test, operator dependent, and IC test, operator independent, might give an acceptable sensibility and confidence level of parasite detection in dairy cattle.

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