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Cryptosporidium parvum Prevalence and Molecular Typing in Dairy Calves with Diarrhea in Córdoba, Argentina

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Abstract

Cryptosporidiosis in calves is caused by the enteroprotozoan pathogen *Cryptosporidium* spp. The disease results in intense diarrhea associated with substantial economic losses in dairy farming worldwide. The objectives of this study were to determine calves, herd and within-herd *Cryptosporidium* prevalence; identify *Cryptosporidium* species and C. *parvum* subgenotypes in calves with diarrhea in intensive dairy herds in central Argentina. A total of 1073 fecal samples were collected from 54 dairy herds randomly selected. Samples were processed with formol-ether to concentrate *Cryptosporidium* oocysts and were detected by light microscopy using modified Ziehl-Neelsen technique. Positive samples were further analyzed to determine the infecting *Cryptosporidium* specie by PCR-RFLP of the 18S rRNA gene resulting in exclusive identification of *Cryptosporidium parvum*. The overall prevalence of C. *parvum* for all calves studied was 25.5% (95% C.I.: 22.9; 28.1%). Sequence analysis of the GP60 gene revealed 5 different subgenotypes (IIaA18G1R1, IIaA20G1R1, IIaA21G1R1, IIaA22G1R1, and IIaA24G1R1), all belonging to the zoonotic IIa family. The most commonly isolated subgenotypes in calves with diarrhea and high infection intensity were IIaA20G1R1 and IIaA18G1R1. The subgenotype IIaA24G1R1 is first reported. The most frequently found subgenotype IIaA18G1R1 in this study is implicated in zoonotic transmission, which combined with a high prevalence suggests that calves might be an important source for human cryptosporidiosis in Argentina.

ABBREVIATIONS

UEL-Villa María (Local Executing Unit); RFLP; PCR

INTRODUCTION

Cryptosporidium is an apicomplexan enteroprotozoan that infects a wide range of vertebrate hosts including animals and humans. The infection is common in calves causing cryptosporidiosis which is characterized by severe diarrhea, lethargy, anorexia, and dehydration. Calves suffering from severe cryptosporidiosis may recover after 4-6 weeks [1] and economic losses due to the infestation are associated with the development of diarrhea and the extra special care cost demanded by diarrheal calves. A mortality of 35.5% in calf less than 30 days old has been reported [2].

Mainly four *Cryptosporidium* species have been reported in cattle *Cryptosporidium parvum, Cryptosporidium bovis, Cryptosporidium andersoni* and *Cryptosporidium ryanae* [3]. Of these, only *C. parvum* is an important zoonotic pathogen, and has been responsible for serious human diarrhea outbreaks both in industrialized and developing countries, affecting especially inmunocompromised individuals and children [4].

Cryptosporidium can be transmitted via fecal/oral route, following direct or indirect contact with infected animal [5,6]. Of the zoonotic source of infection, cattle are recognized as a major contributor, because species and subtypes of *Cryptosporidium* infecting humans have also been isolated from cattle [7]. Therefore, *Cryptosporidium* was associated with increased risk of case death in toddlers aged 12-23 months [8].

High prevalence of *Cryptosporidium* spp. has been reported in dairy calves, with values ranging from 50.5% to 67% [9-

11] or from 77 to 96% [12-16]. It has been described that cryptosporidiosis risk is higher in calves younger than 30 days old [14-16].

Molecular tools have allowed to define and assess the genetic diversity of *Cryptosporidium* facilitating the quest to unravel transmission patterns and associated impacts on public health [17]. In order to assess a possible transmission source *C. parvum* intraspecific subgenotyping based on the 60 kDA glycoprotein (GP60) gene is commonly used [18]. Furthermore, identification of *Cryptosporidium* intraspecific variation has been central in understanding transmission dynamics of the zoonotic species of this parasite.

Molecular characterizations of C. *parvum* at subtype level in dairy calves, important to determine zoonotic potential, have been conducted in several countries [7,15,19-22].

In Argentina, two previous studies have reported altogether 8 different C. *parvum* subgenotypes (IIaA16G1R1 IIaA17G1R1, IIaA18G1R1, IIaA19G1R1, IIaA20G1R1, IIaA21G1R1, IIaA22G1R1, and IIaA23G1R1) in 120 dairy calves [23, 24]. Nevertheless, so far no study has established an association of *C. parvum* subgenotypes and the development of diarrhea in dairy calves. Interestingly, an association between subgenotype and ocurrence of diarrhea has been shown in humans [25-27].

The aim of this study was to determine (i) the prevalence *Cryptosporidium* infection in calves, herds, and within-herd and (ii) the *Cryptosporidium* species and *C. parvum* subgenotypes infecting calves with diarrhea in intensive dairy herds in central Argentina.

MATERIALS AND METHODS

Study area

The study took place in the dairy area located in San Martín, Córdoba, Argentina. In this area there are approximately 564 dairy herds located [28] that represent 68% of the total of dairy herds with 101 to 500 cows [29].

Enrolment of farms

Farms were randomly selected from a database of the Producer's Rural Association, registered in the UEL-Villa María, General San Martín district in Córdoba, Argentina. The inclusion criterion for dairy farms was a herd size of 100 to 300 cows, as based on data of January of 2013. Of the 365 farms that satisfied the inclusion criteria, 60 farms were randomly selected to be studied (Microsoft Excel, 2010).

Sample collection

Farms were visited once between April 2013 and March 2014. At each visit, calves less than 60 days old were sampled. Individual fecal samples were taken directly from the rectum using sterile plastic gloves and stored at 4 to 8°C before transfer to the laboratory.

At the moment of sample collection, the consistency of each fecal sample and the presence of diarrhea were registered. The consistency was scored as follows: solid (S), semisolid (SS), liquid (L), semiliquid (SL), and meconium (M). Diarrhea was defined as runny or liquid feces, with rectal temperature higher than 39.5°C or presence of mucus, fibrin or blood.

Microscopic detection

Samples were processed with the formol-ether concentration technique [30] and presence of *Cryptosporidium* oocysts was verified by microscopic examination of fecal smears stained with the modified Ziehl-Neelsen technique at a magnification of 100 × [31].

The intensity of *Cryptosporidium* infection was scored semi-quantitatively according to the average number of oocysts in 20 randomly selected fields as follows: 0 (no oocysts), 1 (1-5 oocysts), 2 (6-10 oocysts), 3 (11-15 oocysts) and 4 (≥16 oocysts). One person conducted all test.

Molecular determination of species and subgenotype

For species determination genomic DNA isolated from oocyst-positive fecal samples were subjected to PCR-RFLP as described by Tomazic et al. [23], including the use of an additional restriction enzyme, MboII [32].

For subgenotyping *C. parvum* isolates were selected from calves with diarrhea and high infection intensity (score 3 and 4) and the PCR amplicon of the gene encoding the 60kDa glycoprotein (GP60) was directly sequenced and subgenotyped as reported by Tomazic et al. [23]. GP60 subtypes were designated based on the number of trinucleotides TCA and TCG, and the hexanucleotide ACATCA in the polymorphicrepeat region [34]. Nucleotide sequences were deposited in the GenBank database under accession numbers KX768771-KX768816.

Statistical analysis

Overall, herd, and within-herd prevalence of *Cryptosporidium parvum* was calculated for all calves sampled. A chi-square test (χ^2) was used to determine the relationship between animal age and the occurrence of C. *parvum* subgenotype-infection. Subsequently, an ANOVA test was applied to determine the relationship between animal age and the presence of each C. *parvum* subgenotype. All analyses were performed using the statistical software R 3.2.3.

RESULTS AND DISCUSSION

Descriptive data

Of a total of 60 herds selected, 6 did not participated in the study, 3 herds could not be contacted due to incorrect data, 2 herds send their calves to a collective breeding artificial system and 1 herd had sold all animals at the time of the study. A total of 1073 fecal samples were collected in 54 dairy herds. On average 20 samples by herd (range 10 to 66) were sampled. At the time of the study 25% of all calves sampled had diarrhea (95% C.I.: 22.34, 27.06).

Prevalence of Cryptosporidium parvum in calves, herds and within-herd

A total of 282 calves (26.3%) were found positive after microscopic examination of feces using the Zhiel-Neelsen staining technique. Of these 274 genomic DNA was isolated

and subjected to PCR-RFLP in order to determine the infecting species. Exclusively *Cryptosporidium parvum* and no other *Cryptosporidium* species was identified in the study, corroborating previous findings reported in two independent studies [23,24].

The overall prevalence of *C. parvum* for all calves studied was 25.5% (95% C.I.: 22.9; 28.1%). Of the total positive samples: 96 (35%) had an infection intensity of score 1, 113 (41%) score 2, 36 (13%) score 3, and 29 (11%) score four. The overall prevalence is higher than those previously observed in comparable studies in Argentina. This may be due to prior concentration of oocysts from fecal samples which significantly increases the sensitivity of detection [50].

However, the overall prevalence of C. *parvum* in calves is comparable to some studies done in other countries [10-12,35], although there have been higher prevalence values reported [13,16,36]. There are many factors that may be responsible for these prevalence differences, such as management measures, calf age, season and calf housing. Some of these factors may act to increase the risk factors associated with transmission and prevalence of Cryptosporidium between calves [36,37].

Delafosse et al. [16], found that the mortality rate at 90 days of age was higher in calves with high infection intensity score (> 2). Importantly, in our study most positive *C. parvum* calves showed a high infection intensity suggesting that they likewise may suffer an increased mortality.

The overall herd prevalence of *C. parvum* was 89% (at least one *C. parvum* positive calf detected in the herd). Herd prevalence in previous studies is highly variable and can vary from 50.5% to 67% [9-11,38] or from 77 up to 96% [12-16]. These observed differences may be due to an increased sensitivity of diagnostic approach used in this study concentrating oocysts previous to microscopic examination. Furthermore, in the present study, all calves less than 60 days old present in the herds were sampled, increasing the probability of finding at least one positive calf in each herd (Figure 1). In contrast, a low number of calves sampled in each herd may significantly reduce the probability of finding positive calves particularly in herds with a very low prevalence.

While the herd prevalence was high, the within-herd *C. parvum* prevalence was lower, ranging from 0% to 57% (mean 25%; first quartile, 14.7%; third quartile, 36%), (Figure 1), in other countries values found varied from 67% to 90% [10,12,13,16]. This may be due to techniques sensitivity, or with the age of sampled calves, it is known that *C. parvum* prevalence is higher in calves less than 30 days old [19]. In a previous study performed in Córdoba, Argentina, Tiranti et al. [14], observed a similar within-herd prevalence, but no molecular techniques were made that could determine *Cryptosporidium* species, therefore results cannot be compared.

Six (11%) of the 54 herds were negative and conversely in 50% of herds, *C. parvum* within-herd prevalence was higher than average.

In 6 herds, no calves with diarrhea were identified and in 3 of these, no positive *C. parvum* calves were detected. Interestingly, 3 other herds presented only 1 or 2 *C. parvum* positive calves with low infection intensity (score 1). This observation demonstrate that when diarrhea was not commonly observed, no or very low *C. parvum* infection was detected, which demonstrates the significance of *C. parvum* as one of the most important enteric pathogens causing diarrhea in neonatal dairy calves [11,39,40].

Cryptosporidium parvum subtyping

Of the 65 *C. parvum* positive calves and with high infection intensity (score 3 and 4), 47 DNA isolates from calves with diarrhea at the time of sampling were selected. Of these forty-seven samples genomic DNA was isolated and the C. *parvum* GP60 gene sequence amplified and directly sequenced. No sample contained more than one C. *parvum* GP60 subgenotype, and all sequences belonged to the IIa family that includes also

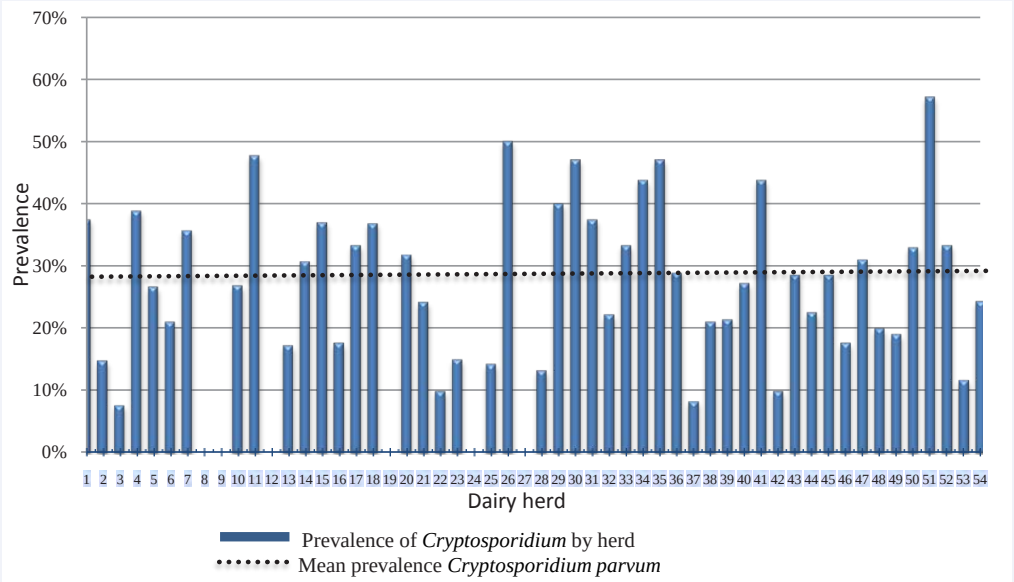

Figure 1 Prevalence of *Cryptosporidium parvum* by dairy herd (n=54).

zoonotic variants.

Five *C. parvum* GP60 subtypes (IIaA18G1R1, IIaA20G1R1, IIaA21G1R1, IIaA22G1R1 and IIaA24G1R1) were identified (Table 1). Remarkably, as in a previous study we observed in all analyzed alleles a non-synonymous nucleotide exchange from "GAC" to "GGC" resulting in an exchange of Asn to Gly at amino acid site 99. Thus, all GP60 sequences isolated so far from calves in Argentina present this non-synonymous mutation suggesting genetic drift [23,24].

The most predominant subtype found in calves with diarrhea and high infection intensity was IIaA20G1R1 (25/47), followed by IIaA18G1R1 (12/47). Importantly, an association between subgenotype and diarrhea was found in a study carried out in Poland [22]. In this study 8 calves infected by the *C. parvum* subgenotype IIaA16G1R1b were identified, all of which presented diarrhea.

A novel subgenotype IIaA24G1R1 not previously reported could be identified in this study. IIaA24G1R1 could be isolated from two different calves that belonged to the same herd. These two animals showed profuse diarrhea and a high-level of dehydration, and, though treated, one of them died from diarrhea, and laboratory investigations could exclude bacterial infections as a primary cause of death (personal communication). Besides the novel IIaA24G1R1 subgenotype, the remaining 4 subgenotypes reported in this study have been previously detected in Argentina [23,24].

The most predominant subtype in calves with diarrhea, IIaA20G1R1, was previously reported in Argentina [23,24], Sweden [21], Serbia and Montenegro [42]. In other studies carried out in Argentina the IIaA20G1R1 subgenotype was also found with relatively high frequency (8 of 46), [23] and (27 of 75), [24], suggesting that it may be the prevalent subtype in this country (Table 1).

The second most frequent subgenotype identified in the study (IIaA18G1R1), seems to be more widely distributed as it has also been reported in England [43], The Netherlands [44], Sweden [21], Czech Republic [45], Hungary [46], Serbia and Montenegro [42], Brazil [47], and Argentina [23,24] (Table 1). In a study done in England, this subgenotype was also identified in humans [48] strongly suggesting that there is a risk of zoonotic transmission. In a recent study carried out in Argentinean and Brazilian human subjects, *C. hominis* was the species more frequently detected [48] and none of the C. parvum subgenotypes reported in this study were found.

No statistically significant relationship observed between calves age and the presence of a specific subtype ($p=0.49$, $\alpha > 0.05$). Nevertheless, particular subtypes were observed only in animals of a certain age. For example, IIaA21G1R1, IIaA22G1R1 and IIaA24G1R1 subtypes were observed in the youngest animals of less than two weeks of age. In contrast, IIaA21G1R1 and IIaA24G1R1 subgenotypes where isolated only in calves of two weeks of age and the IIaA18G1R1 subgenotype was found exclusively in calves older than two weeks of age (Figure 2).

Table 1: *Cryptosporidium parvum* GP60 subgenotypes in calves from Córdoba, Argentina.

Subgenotype	Samples by age groups [a]				Total	Country [b]	Reference
	1 week	2 weeks	3 weeks	4 weeks			
IIaA18G1R1	0	5	6	1	12	Argentina (1 of 46; 13 of 75)	[23,24]
						England (2 of 51)	[43]
						The Netherlands (2 of 129)	[44]
						Sweden (3 of 171)	[21]
						Czech Republic (3 of 137)	[45]
						Hungary (1 of 21)	[46]
						Serbia and Montenegro (2 of 18)	[42]
						Brazil (1 of 28)	[47]
IIaA20G1R1	1	16	7	1	25	Argentina (8 of 46; 27 of 75)	[23,24]
						Sweden (5 of 171)	[21]
						Serbia and Montenegro (2 of 18)	[42]
IIaA21G1R1	0	2	0	0	2	Argentina (15 of 46; 10 of 75)	[23,24]
						Sweden (11 of 171)	[21]
IIaA22G1R1	1	5	0	0	6	Argentina (5 of 46; 16 of 75)	[23,24]
						Sweden (7 of 171)	[21]
						Germany (1 of 53)	[50]
						Czech Republic (12 of 137)	[45]
IIaA24G1R1	0	2	0	0	2	None	None
Total	2	30	13	2	47		

[a] Frequency of samples by age groups and *C. parvum* subgenotypes.
[b] Country in which the respective subgenotype has been reported in calves. Number of positive samples and total examined is shown.
Abbreviations: UEL-Villa María: Local Executing Unit Villa María City; RFLP: Restriction Fragment Length Polymorphism.

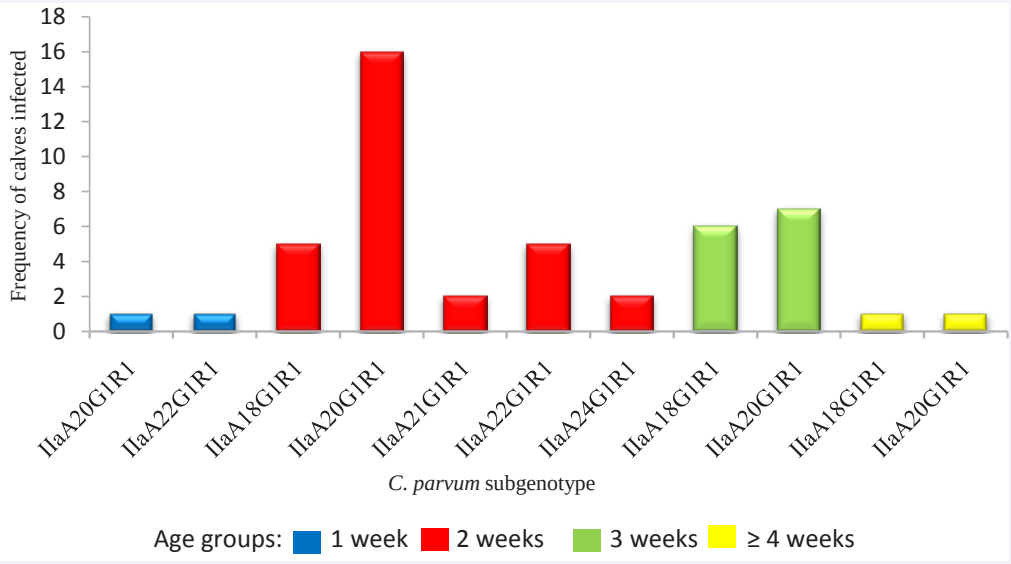

Figure 2 Frequency of *C. parvum* subgenotypes and calves age.

Importantly, the most predominant subgenotype IIaA20G1R1, was identified in all age groups and seems to be widely distributed as it has been reported from other regions of Argentina [23,24] (Figure 2).

Although a high prevalence was observed in the present study, this might be even underestimated because of the examination of a single fecal sample from each calf. In addition, possible co infections with other microorganisms was not evaluated, therefore *C. parvum* association with diarrhea may be overestimated.

Further research is required in order to understand the real clinical significance of each *C. parvum* subgenotype, as well as the interaction with co-infections. The presence of at least one *C. parvum* zoonotic subgenotype in dairy calves may suggest the implications for transmission to humans. Consequently, molecular epidemiology studies are necessary in humans, especially in workers of these herds to better understand the distribution, zoonotic potential and transmission routes.

CONCLUSIONS

The current study revealed that *Cryptosporidium parvum* is widely distributed in dairy calf operations in the area studied. Five *C. parvum* GP60 subtypes (IIaA18G1R1, IIaA20G1R1, IIaA21G1R1, IIaA22G1R1 and IIaA24G1R1) were identified. The most commonly isolated subgenotypes in calves with diarrhea and high infection intensity were IIaA20G1R1 and IIaA18G1R1. A new subtype IIaA24G1R1was identified. No statistically significant relationship was observed between the age of calves and the presence of a specific subgenotype.

The most frequently subgenotype (IIaA18G1R1) found in this study is strongly implicated in zoonotic transmission. This combined with the high prevalence found suggests that calves might be an important source for human cryptosporidiosis in Argentina. Additional studies including humans are required.

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