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Prevalence, risk factors and molecular epidemiology of neonatal cryptosporidiosis in calves: The Argentine perspective

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ABSTRACT

Cryptosporidium spp. are enteroparasitic protozoans that cause cryptosporidiosis in newborn calves. Clinical signs of the infection are diarrhoea and dehydration leading to decreased productivity and economic losses in cattle farms around the world. Additionally, cryptosporidiosis is a relevant zoonotic disease since the ingestion of oocysts can be fatal for children under five years of age, the elderly, and/or immunocompromised adults. This review aims to integrate existing knowledge on the epidemiological situation of calf cryptosporidiosis and associated risk factors in Argentina. In addition, the GP60 subtype diversity of the pathogen was analysed and related with the global distribution of corresponding GP60 subtypes. Depending on the study region and applied diagnostics, prevalence among calves up to 20 days of age varied between 25.2% and 42.5%, while a prevalence of 16.3–25.5% was observed at the age of 1–90 days. So far, molecular studies have determined exclusively *Cryptosporidium parvum* in preweaned calves. In addition, *C. parvum* infection was reported as the major cause of calf diarrhoea, followed by rotavirus A (RVA), while enteropathogens such as coronavirus, *Escherichia coli*, and *Salmonella* sp. played a negligible role. Calf age of 20 days or less, incidence of diarrhoea, poorly drained soils, and large farm size were identified as risk factors for *C. parvum*-infection in Argentina. A total of nine GP60 subtypes (IIaAxxG1R1, xx = 16 to 24) were identified, showing a stepwise increase of the trinucleotide motif TCA, and including the zoonotic subtypes IIaA16G1R1, IIaA17G1R1, IIaA18G1R1, IIaA19G1R1, and IIaA20G1R1. We found that an increase in the A16→A24 trinucleotide repeat was accompanied by a gradual decrease in the global distribution of GP60 alleles, strongly suggesting that IIaA16G1R1 represents the primordial allelic variant of this group. Since identified GP60 alleles have a similar genetic background, we hypothesize that the continuous trinucleotide repeat array has been generated by stepwise repeat expansion of A16. The information gathered and integrated in this study contributes to an improved understanding of the epidemiological characteristics of bovine cryptosporidiosis in and beyond Argentina, which in turn can help to develop control strategies for this parasitosis of veterinary and medical relevance.

1. Overview of bovine cryptosporidiosis

Infectious diarrhoea of neonatal calves caused by enteropathogens is an ongoing concern for dairy production systems worldwide (Foster and Smith, 2009; Vermeulen et al., 2017; Tomazic et al., 2018; Chen et al., 2023). Currently, there are no effective prevention and treatment methods, and the costs related to veterinary care of affected animals are high. Diarrhoeal infections lead to dehydration, resulting in reduced growth rates in calves. Additionally, the outcome can be fatal, and about half of the deaths of calves under 8 weeks of age on dairy farms are due to diarrhoea, as reported in the USA and South Korea, as well as other countries (Sanford and Josephson, 1982; USDA, 2008; Foster and Smith, 2009; Hur et al., 2013).

Different pathogens such as rotavirus A (RVA) and coronavirus, the bacteria *Escherichia coli* and *Salmonella* spp. and the protozoan *Cryptosporidium* spp. cause bovine neonatal diarrhoea (Foster and Smith, 2009; Tomazic et al., 2018). Among these, *Cryptosporidium* spp. are worldwide distributed and represent the most frequent pathogenic agent identified in calves. For example, in one case, an infection rate of up to 84.0% was reported in a dairy herd in Argentina (Modini et al., 2010).

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Cryptosporidium spp. are parasitic protozoans of the phylum Apicomplexa that infect mammals, reptiles, amphibians, birds, and fish (Fayer, 2010). In cattle, four species are principally detected: *C. parvum*, *C. bovis*, *C. ryanae*, and *C. andersoni* (Xiao and Feng, 2008; Xiao, 2010; Tomazic et al., 2018). *Cryptosporidium parvum* is considered the predominant species in calves up to 2 months of age (Santín et al., 2008; Follet et al., 2011; Amer et al., 2013; Tomazic et al., 2018), but, in a few cases, infection with *C. bovis* in this age group has also been reported in Sweden, China, Canada, India, and the USA (Feng et al., 2007; Silverlås et al., 2010; Budu Amoako et al., 2012; Wang et al., 2017). Notwithstanding, only *C. parvum* is considered pathogenic, representing the principal causative agent of calf scours, characterised by diverse degrees of diarrhoea, anorexia, and abdominal pain, and which may lead to death (Nydam et al., 2001; Thompson et al., 2008; Tomazic et al., 2013; Del Coco et al., 2014; Cho and Yoon, 2014; Lombardelli et al., 2019; Garro et al., 2021; Bertoni et al., 2021).

Cryptosporidium spp. can also cause moderate to severe self-limiting diarrhoea in healthy human individuals. Most cases are caused by the zoonotic *C. parvum* or the anthroponotic *C. hominis*. However, in immunocompromised patients, such as those with AIDS, hemo-oncological diseases, and transplantations, the infection is associated with chronic and life-threatening diarrhoea. In addition, *Cryptosporidium* spp. have been shown to be a major cause of mortality in children under 2 years of age in sub-Saharan Africa and South Asia, and have recently been recognized as an important cause of child malnutrition and a health problem for the elderly around the world (Xiao and Feng, 2008; Checkley et al., 2015; Sow et al., 2016; Yang et al., 2021). Human infection with *C. parvum* subtype IIa is more common in developed countries, particularly in Europe, than in developing countries. This may be due to the predominance of subtype IIa infection in intensive cattle production systems in these countries resulting in massive oocyst contamination of the environment (Guo et al., 2022). In contrast, the prevalence of *C. parvum* subtype IIc and *C. hominis* is higher in developing countries, because of direct human contact, inadequate hygiene, lack of clean water, and poor sanitation facilities (Feng et al., 2007; Gerace et al., 2019; Yang et al., 2021). Increasingly, molecular PCR-based diagnostics results in an improved identification and documentation of the infecting species and subtypes (Xiao, 2010; Xiao and Feng, 2017; Guy et al., 2021). Few human cases of cryptosporidiosis have been reported in Argentina, possibly due to the lack of accurate diagnosis (Cerezuela et al., 2017).

Cryptosporidium parasites have a monoxenic life-cycle with asexual and sexual reproduction. The infected animal excretes with its faeces sporulated oocysts into the environment. Transmission to a new host occurs *via* the faecal-oral route, either by direct contact with infected faeces or indirectly by consuming water or food contaminated with oocysts. After ingestion by the host, the oocysts excyst and invade the cells of the gastrointestinal tract where they undergo asexual and sexual reproduction, during which thin-walled and thick-walled oocysts are generated. The former generate sporozoites that autoinfect yet uninfected intestinal cells, while the latter are excreted into the environment with faeces (Thompson et al., 2008; Bouzid et al., 2013; Tomazic et al., 2018).

The evolution of *Cryptosporidium* spp. has resulted in extremely efficient host colonization, parasite proliferation, and dissemination strategies. Successive multiplication and autoinfective cycles in the host result in a massive amplification of the parasite in the digestive tract, ensuring an efficient excretion of a huge number of oocysts into the environment. It has been reported that a single calf excretes up to 40 million oocysts per gram of faeces, corresponding to 600 million oocysts per day (Fayer et al., 1998; Zambriski et al., 2013). Moreover, the thick oocyst wall allows resisting a wide variety of environmental conditions and many disinfectants, including those based on chlorine. Finally, an extremely low infective dose ensures dissemination in the host population: it has been demonstrated that ingestion of only 17 oocysts allows the establishment of infection in calves (Nydam et al., 2001; Messner and Berger, 2016). These characteristics ensure the completion of the life-cycle and parasite survival. However, they also result in massive oocyst contamination of the environment, especially near livestock production facilities (Thompson et al., 2008; Wyatt et al., 2010).

Among several available methods for the detection of *Cryptosporidium* oocysts in faeces, the most widespread is the microscopic examination of acid-fast stained oocysts in faecal smears using methods such as Kinyoun and modified Ziehl-Neelsen. These methods take advantage of the presence of fatty acids in the oocyst wall allowing to clearly spot *Cryptosporidium* oocysts as small pink spheres against a blue background (Henriksen and Pohlenz, 1981; Casemore et al., 1985; Petry, 2004; Jenkins et al., 2010; Aldeyarbi and Karanis, 2016; Tomazic et al., 2018; Wang et al., 2022). Noteworthy, a less common, but faster, more specific, and more sensitive method is the modified Heine negative staining (Potters and Van Esbroeck, 2010; Khanna et al., 2014). Although rapid, staining methods are less sensitive than molecular diagnostics and do not allow species differentiation (Santin, 2020).

In order to identify the infecting *Cryptosporidium* species, demonstration of species-specific DNA in stool or environmental samples is required (Chalmers and Katzer, 2013; Santin, 2020). Current molecular methods are mainly based on the detection of species-specific polymorphisms in the variable region of the 18S small subunit ribosomal RNA (18S rRNA) gene. Differentiation between species is made possible by length analysis of the amplicon after species-specific PCR or by PCR followed by enzymatic digestion of the amplicon (PCR-RFLP) (Xiao, 2010; Thomson et al., 2016; Xiao and Feng, 2017; Santin, 2020). In addition, subtyping of *C. parvum* can be carried out by PCR amplification and sequencing of the gene encoding the polymorphic 60 kDa glycoprotein (GP60) (Xiao, 2010). Notably, some *C. parvum* GP60 subtypes have been associated with zoonosis (Thompson et al., 2008; Xiao, 2010). Other genotyping methods have been developed, but their complexity limits their use to specific research questions (Widmer et al., 2004; Díaz et al., 2012). Analysis of the molecular epidemiology of the subtypes of *Cryptosporidium* allows to study the transmission dynamics of the infection, the sources of contamination, and the zoonotic risk, and is therefore of great importance both for the surveillance of the agricultural production system and for public health.

At present, there are neither vaccines nor efficient chemotherapeutics available for the control of bovine cryptosporidiosis (Tomazic et al., 2018; Santin, 2020; Florin-Christensen et al., 2021). Therefore, increased knowledge of this parasitosis, its prevalence and associated risk factors is required in order to develop and improve current strategies to control the spread of the parasite. This review aims to analyse and integrate different aspects of the prevalence, associated risk factors, and molecular epidemiology of *Cryptosporidium* spp. infection in cattle in Argentina.

2. Prevalence of *Cryptosporidium* spp. infection of dairy calves

The presence of *Cryptosporidium* oocysts in calves with clinical signs of diarrhoea in dairy herds was first reported in several Argentine provinces by Bellinzoni et al. (1990) who observed a very high infection rate of 29.6% in dairy calves less than 30 days of age. However, this value needs to be interpreted with caution. On one hand, only calves with clinical signs were included in this study, and on the other hand, faecal samples were not concentrated before microscopic examination. Thus, although the observed infection rates indicate that *Cryptosporidium* infection is important and widespread in calves in Argentina, the reported values represent the infection rate of diarrhoeic calves and should therefore not be considered as prevalence (Bellinzoni et al., 1990) (Table 1).

In more recent studies, the prevalence of *Cryptosporidium* spp. infection has been determined in five extensive and well-projected epidemiological regional studies including a large number of animals sampled from many dairy farms ($n \leq 19$ to 54 dairy herds) in the provinces of Buenos Aires, Córdoba, and Salta (Garro et al., 2016, 2021;

Table 1
Epidemiology of *Cryptosporidium* infection in dairy calves in Argentina.

Province	Farms (*n*)	Samples (*n*)	Prevalence (%)[a]			Sample processing (Concentration method)[b]	Microscopic examination (Staining method)[b]	Reference
			Calf-level		Herd-level			
			Age ≤90 days	Age ≤20 days				
Buenos Aires	42	908	18.5	29.8	73.8	Formalin-ether[1]	Kinyoun[4]	Garro et al. (2021) [c]
	27	552	16.3	26.6	98.0	None	Kinyoun[4,6]	Garro et al. (2016)
	1	280	17.0	24.0	–	Water/ether[3]	Modified Ziehl-Neelsen[5]	Del Coco et al. (2008)
Córdoba	54	1073	25.5	34.0	89.0	Formalin-ether[1]	Modified Ziehl-Neelsen[4]	Lombardelli et al. (2019) [c]
	43	620	19.4	25.2	79.0	Formalin-ether[1]	Modified Ziehl-Neelsen[4]	Tiranti et al. (2011)
Santa Fe	*8*	*135*	*19.9*	*46.2*	–	Sheather[2]	Kinyoun[4]	Aguirre et al. (2014)
	1	*106*	*84.0*	*83.0*	–	Sheather[2]	Kinyoun[4]	Modini et al. (2010)
	3	*162*	*24.0*	*43.0*	*100*	Sheather[2]	Kinyoun[4]	Modini et al. (2011)
Salta	19	488	18.0	42.5	84.2	Water[4]	Modified Ziehl-Neelsen[4]	Bertoni et al. (2021)
Five province study[d]	*33*	*239*	–	*29.6*	*69.7*	None	Bronsdon[7]	Bellinzoni et al. (1990)

[a] Studies that estimated the true prevalence across a large region including at least 19 herds are shown in normal font type, while studies that estimated the infection rate based on one to eight farms are displayed in italics.
[b] The references for the concentration and staining methods applied are: [1]Young et al. (1979); [2]Garcia (2007); [3]Bukhari and Smith (1995); [4]Henriksen and Pohlenz (1981); [5]Casemore et al. (1985); [6]Elsafi et al. (2014); [7]Bronsdon (1984).
[c] In these studies, the species *C. parvum* was identified in a subset of oocyst-positive samples using PCR-RFLP.
[d] Exclusively faecal specimens from calves under 30 days of age with clinical signs of diarrhea were sampled from farms situated in the Province of Buenos Aires, Córdoba, Santa Fe, Entre Rios, and La Pampa.

Tiranti et al., 2011; Lombardelli et al., 2019; Bertoni et al., 2021) (Fig. 1, Table 1). In two independent studies carried out in different extended regions of the Buenos Aires Province, a similar prevalence of 26.6% and 29.8% was found in calves ≤ 20 days of age. However, the first value may represent an underestimation since oocyst concentration was not applied (Table 1; Garro et al., 2016, 2021). Interestingly, a similar infection rate of 24.0% oocyst-excreting calves from a single dairy herd has been also reported by Del Coco et al. (2008) (Table 1). In the same

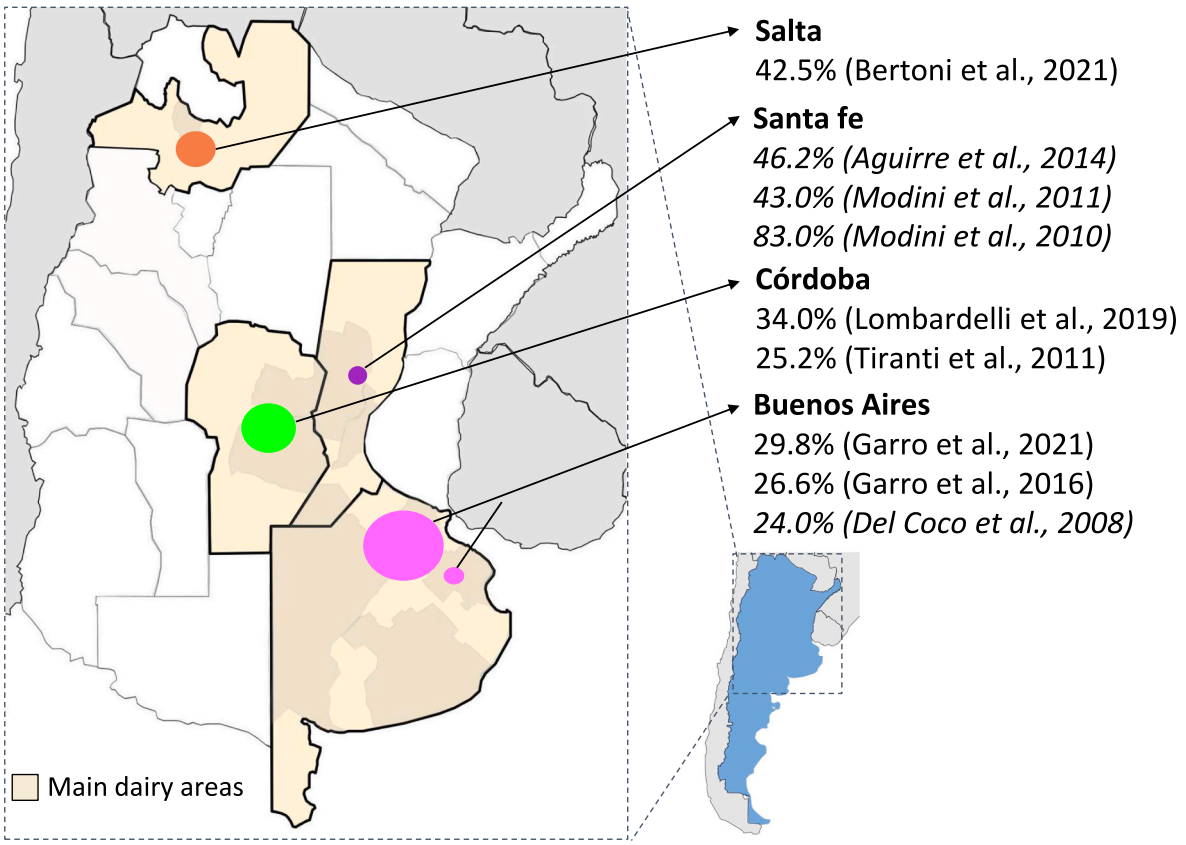

Fig. 1. Prevalence of oocyst-excreting calves younger than 2 years of age sampled from herds in different geographical regions in Argentina. Circle size approximately corresponds to the number of sampled herds and the size of the studied region. Values and references in italics are estimates based on single or few herds and represent infection rates of the individual farms studied (Table 1).

age group of calves, two studies from the same regions of the Córdoba Province reported a prevalence of 25.2% and 34.0% (Tiranti et al., 2011; Lombardelli et al., 2019). Since the same diagnostic methodology was applied, the difference demonstrates an important increase in the prevalence during this time. Since a similar number of samples were taken throughout the year in both studies, the observed differences are not due to seasonality. Noteworthy, between 2011 and 2019 production had been substantially intensified in this region, and animal density in breeding sectors had doubled possibly resulting in increased contact of animals facilitating the spread of the parasite (J. Lombardelli, pers. comun.). Large farm size and an increased stocking density have been reported as significant risk factors for *Cryptosporidium* infection (see *Section* 4) (Guo et al., 2022; Chen et al., 2023). Interestingly, a considerably higher prevalence of 42.5% in calves ≤ 20 days of age was reported from Salta Province. However, the elevated value may be due to an increased concentration procedure during processing of faecal samples prior to microscopic examination, resulting in a higher prevalence value in this region (Table 1; Bertoni et al., 2021). Except for one study from Córdoba, where 25.5% of calves under 90 days of age were identified to excrete oocysts, a considerably lower prevalence, ranging between 16.3% and 19.4%, was observed in this age group in studies reported from Córdoba, Buenos Aires, and Salta (Tiranti et al., 2011; Garro et al., 2016, 2021; Lombardelli et al., 2019; Bertoni et al., 2021).

Other epidemiological investigations carried out in Santa Fe Province included a considerably lower number of calves sampled from a single or up to eight dairy herds (Table 1). In addition, these studies stay apart because of the use of the highly sensitive Sheather's concentration method, which may explain in part the considerably higher infection rates of 43% and 46.2% observed in calves under 20 days of age, as reported by Modini et al. (2011) and Aguirre et al. (2014), respectively (Table 1). Furthermore, an unusually high rate of infection of 83.0% in calves under 15 days of age, and of 85% in calves under 60 days of age were reported from a single farm that artificially raised dairy calves from other farms. This procedure is expected to significantly increase the infection pressure on susceptible animals by introducing, accumulating, and contaminating oocysts. Finally, depending on the region, the prevalence of *Cryptosporidium* infection in young calves ≤ 20 days of age in Argentina was found to range from 25.2% to 42.5%, being lower than that reported from Galicia, Spain (47.9%), in a similar range as those reported from Latvia (33.8%), North-West England (28%) and The Netherlands (27.8%), but higher than that reported from New Zealand (15.8%). All of the referenced studies used direct microscopic examination of oocysts after staining or immunofluorescence, and although sensitivities may vary to some extent, the reported prevalences should be comparable with a reasonable degree of caution. This comparison shows that the prevalence rates reported from Argentina are significant and place this country in the upper middle range of the countries surveyed (Castro-Hermida et al., 2002; Brook et al., 2008; Bartels et al., 2010; Al Mawly et al., 2015; Deksne et al., 2022; Guo et al., 2022).

3. Diarrhoea as a clinical manifestation of *Cryptosporidium* spp. infection

Cryptosporidium infection is associated with diarrhoea as a clinical manifestation in calves less than 21 days of age (OR = 5.7, Garro et al., 2021) or 60 days of age (OR = 3, Bertoni et al., 2021; OR = 5.5, Garro et al., 2016), as reported in large-scale studies involving more than 19 herds. Comparable values have been reported for calves of the same age groups when single or few farms were sampled and studied (RR = 5.9, Aguirre et al., 2014; RR = 2.9, Modini et al., 2011) (Table 2). Garro et al. (2021) showed that, in addition of *Cryptosporidium* (OR = 5.7, $P < 0.0001$), RVA infection (OR = 2.5, $P < 0.05$) was a significant risk factor for diarrhoea in calves ≤ 20 days of age in the Province of Buenos Aires. However, based on these data, it can be concluded that *Cryptosporidium* infection is clearly the predominant causative factor of diarrhoea in the study region, considering the 2-fold higher prevalence of *Cryptosporidium* infection (29.8% *vs* an infection rate of 12.4% for RVA) and an approximately 2-fold higher OR (5.7 *vs* an OR of 2.5 for RVA) for diarrhoea. Both values translate into an approximately 4-fold greater impact of *Cryptosporidium* on the prevalence of diarrhoea in newborn calves. Interestingly, mixed infections with *Cryptosporidium* and RVA increased substantially the risk of developing diarrhoea (Table 2; OR = 9.2, Garro et al., 2021), yet the low number of animals with co-infections observed suggested that this factor contributes only marginally to the overall prevalence of calf diarrhoea (Garro et al., 2021). Noteworthy, the low incidence of *Cryptosporidium* and RVA co-infection is due to the conditional probability of its occurrence, highlighting that *Cryptosporidium*-infected calves do not show an increased susceptibility to RVA infection and *vice versa*.

4. Risk factors for *Cryptosporidium* spp. infection of dairy calves

Cryptospordium infection was found to be highly associated with an age of ≤ 21 days (Table 3; OR = 7.9, Garro et al., 2016; OR = 5.6, Garro et al., 2021; OR = 4.4, Bertoni et al., 2021) or under 15 days of age (RR = 3.8, Tiranti et al., 2011). Somewhat decreased values were reported when lower numbers of animals and fewer farms were sampled (RR = 3.6, Aguirre et al., 2014). The findings are in agreement with research carried out in Spain, Mexico and Canada where a higher risk of *C. parvum* infection was reported in calves in the first two weeks of life compared to age groups from 16 to 60 days (de la Fuente et al., 1999; Maldonado-Camargo et al., 1998; Wade et al., 2000; Maddox-Hyttel et al., 2006; Trotz-Williams et al., 2007; Brook et al., 2008; Cardoso et al., 2008; Santín et al., 2008; Izzo et al., 2011). The high prevalence of oocyst excretion in calves ≤ 20 days of age strongly suggests that this is the most relevant age group to establish controls to prevent contamination of the environment with *C. parvum*, such as, for example, separation of infected calves from the herd (Xiao et al., 2007).

Interestingly, Bertoni et al. (2021) found a slightly increased though highly significant risk of *Cryptosporidium* infection in farms with more than 300 milking cows (Table 3; OR = 1.2, $P < 0.005$). Possibly, lower sanitary conditions in larger farms with more intensive production contribute to the observed higher infection rate compared to farms of

Table 2
Diarrhoea as a clinical manifestation of *Cryptosporidium* spp. infection.

Independent variable	Dependent variable	Levels[a]	OR	RR	95% CI	*P*-value	Reference
Cryptosporidium spp.	Diarrhoea	≤ 20 days	5.7	–	3.3–9.9	<0.001	Garro et al. (2021)
		1–60 days	3	–	1.8–5.0	<0.001	Bertoni et al. (2021)
		1–60 days	5.5	–	2.6–11.6	<0.0001	Garro et al. (2016)
		≤ 21 days	–	*5.9*	*1.3–27.3*	*<0.01*	Aguirre et al. (2014)
		≤ 15 days	–	*2.9*	*1.4–5.6*	–	Modini et al. (2011)
Cryptosporidium + Rotavirus	Diarrhoea	≤ 20 days	9.2	–	2.8–29.0	0.001	Garro et al. (2021)

Abbreviations: OR, odds ratio; RR, relative risk; CI, confidence interval.

[a] Values obtained in studies of three and eight herds are given in italics; values based on regional studies that involved at least 19 or up to 42 herds are given in normal font type (see Table 1).

Table 3
Risk factors associated with *Cryptosporidium* spp. infection.

Independent variable	Dependent variable	Levels[a]	OR	RR	95% CI	*P*-value	Reference
Age	*Cryptosporidium*	≤ 20 days	5.6	–	3.2–9.5	<0.001	Garro et al. (2021)
		< 20 days	4.4	–	2.7–7.2	<0.001	Bertoni et al. (2021)
		≤ 20 days	7.9	–	3.7–17.0	<0.0001	Garro et al. (2016)
		≤ 15 days	–	3.8	2.3–6.3	–	Tiranti et al. (2011)
		≤ 21 days	–	*3.6*	*2.6–5.0*	*<0.01*	Aguirre et al. (2014)
Farm size ($n > 300$)	*Cryptosporidium*	1–60 days	1.2	–	0.5–2.7	0.005	Bertoni et al. (2021)
Poorly drained soils	High prevalence herd	1–50 days	–	2.9	–	0.001	Tiranti et al. (2011)

Abbreviations: OR, odds ratio; RR, relative risk; CI, confidence interval.
[a] Values obtained in studies of eight herds are given in italics; values based on regional studies that involved at least 19 or up to 43 herds are given in normal font type (Table 1).

medium or smaller size (Bertoni et al., 2021). Besides, the heavy environmental contamination generated by such concentrated animal feeding operations (CAFO), they promote propagation and subsequent spillover effects of the pathogen into the environment and then to other farm animals and humans. For example, it has been observed that the same *C. parvum* subtypes found in cattle subjected to intensive farming practices typical for developed countries are identified in human patients with cryptosporidiosis. On the other hand, these subtypes are not identified in bovines and in human patients in developing countries that typically lack CAFOs (Guo et al., 2022).

Finally, Tiranti et al. (2011) analysed the prevalence of *Cryptosporidium* infection in regions of poorly and well-drained soils. They found an almost 3-fold increase of the risk of *Cryptosporidium* infection as a consequence of poorly drained soils (Table 3; RR = 2.9, Tiranti et al., 2011). These results are of great importance as they demonstrate the relevance of the rearing environment for *C. parvum* infection. Noteworthy, Castro-Hermida et al. (2002) observed that the risk of infection was significantly higher in pens with straw soils rather than cement floors. The latter can be easily cleaned with water and disinfected as opposed to straw soils, which need to be periodically changed which complicates disinfection.

5. Molecular epidemiology of *Cryptosporidium* species and subtypes

Molecular characterisation of *C. parvum* subtypes is important to determine associated risk factors and to establish possible infection sources and routes of transmission (Xiao et al., 1999; Xiao, 2010). To date, all oocyst samples isolated in Argentina ($n = 393$) have been identified exclusively as *C. parvum* by PCR-RFLP of the 18S rRNA gene (Tomazic et al., 2013; Del Coco et al., 2014; Lombardelli et al., 2019; Garro et al., 2021). Thus, it is reasonable to assume that prevalence values in the study regions given in Table 1 correspond to the species *C. parvum*. In contrast, in the neighbouring country Brazil, also the presence of *C. bovis*, *C. ryanae*, and *C. andersoni* has been reported in calves under 2 months of age as determined by sequencing of 18S rRNA amplicons (Meireles et al., 2011; Toledo et al., 2017).

Furthermore, all 168 GP60 subtypes analysed so far were found to belong to the family IIa (Tomazic et al., 2013; Del Coco et al., 2014; Lombardelli et al., 2019) (Table 4). The most frequent subtype identified was IIaA20G1R1 ($n = 60$). Other GP60 subtypes that were frequent and evenly distributed in the three geographical study regions were IIaA18G1R1 ($n = 26$), IIaA21G1R1 ($n = 27$), and IIaA22G1R1 ($n = 27$) (Tomazic et al., 2013; Del Coco et al., 2014; Lombardelli et al., 2019). These four subtypes were also detected, although with relatively low frequency, in neonatal calves from many different countries from western and eastern Europe, South America (Uruguay and Brazil), and Middle East (Iraq) (Fig. 2, Supplementary Tables S1 and S2).

Less common subtypes identified in Argentina are IIaA19G1R1 ($n = 1$), followed by IIaA24G1R1 ($n = 2$), and IIaA16G1R1 ($n = 4$). Noteworthy, variants IIaA23G1R1 ($n = 11$) and IIaA24G1R1 ($n = 2$) have been first identified in calves in Argentina but meanwhile the former has been also identified in Sweden and Uruguay, while the latter has been recently identified in Germany (Fig. 2, Supplementary Tables S1 and S2) (Tomazic et al., 2013; Lombardelli et al., 2019). The worldwide most widespread subtypes IIaA16G1R1, IIaA17G1R1 ($n = 10$), and IIaA19G1R1 ($n = 1$), were reported from many countries of western and eastern Europe and from South America (Argentina and Uruguay). In addition, the subtype IIaA16G1R1 was found in North America (Canada), Middle East, Africa, and Australia, whereas IIaA17G1R1 was identified in Middle East and Africa. Finally, IIaA19G1R1 was also distributed in North America and Asia (Fig. 2, Supplementary Table S1).

Statistical analysis applying the Kruskal-Wallis test showed that the number of GP60 subtypes IIaA21G1R1 and IIaA20G1R1 identified in Argentina is significantly higher than that of subtypes IIaA16G1R1, IIaA17G1R1, IIaA19G1R1, and IIaA24G1R1 ($P < 0.05$) (Supplementary Fig. S1). No other statistically significant relations could be demonstrated (Supplementary Data S1).

Association studies of the severity of calf cryptosporidiosis with GP60 variants are scarce. In two recent studies, no association between the severity of diarrhoea and a particular subtype could be observed; however, the sample number was relatively low, and subtypes were not found to be equally distributed but clustered between farms (Del Coco et al., 2014; Lombardelli et al., 2019).

Remarkably, all identified GP60 alleles exhibited a nonsynonymous nucleotide substitution (A → G) in a highly conserved region resulting in an amino acid exchange, from Asp to Gly; an observation that suggests a founder effect (Tomazic et al., 2013). Furthermore, the 5' terminus of the gene contains a polymorphic microsatellite region composed of 16–24 trinucleotide repeats "TCA" (A16 to A24) and a single copy of "TCG" (G1) and "ACATCA" (R1). This observation suggests that *C. parvum* varieties isolated in Argentina evolved from an introduced

Table 4
Cryptosporidium parvum GP60 subtypes identified in calves from Argentina.

Province	IIaXXG1R1 (xx = A16-A21)									No. of subtypes analysed
	A16	A17	A18	A19	A20	A21	A22	A23	A24	
Buenos Aires	4^{2}	10^{1}	13^{2}	1^{2}	$31^{1,2}$	$19^{1,2}$	$18^{1,2}$	$5^{1,2}$		101
Santa Fe					1^{1}	3^{1}	3^{1}	6^{1}		13
Córdoba			$13^{1,3}$		$28^{1,3}$	$5^{1,3}$	6^{3}		2^{3}	54
Total	4	10	26	1	60	27	27	11	2	168

Note: GP60 subtypes have been reported in [1]Tomazic et al. (2013) ($n = 46$), [2]Del Coco et al. (2014) ($n = 75$); [3]Lombardelli et al. (2019) ($n = 47$).

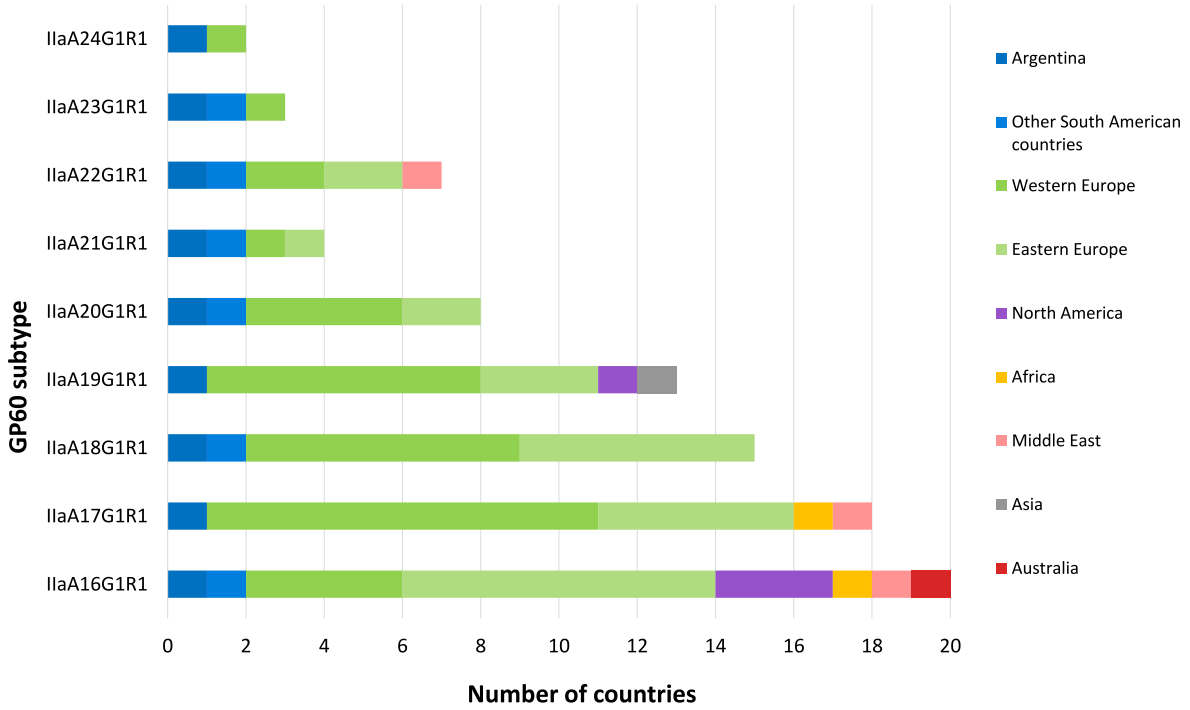

Fig. 2. GP60 subtypes IIaAxxG1R1 (xx = 16 to 24, the figures correspond to the number of repeats of the trinucleotide GTA) reported from Argentina and their global geographical distribution. Colours indicate countries or geographical regions where a defined subtype was found. Argentina (*blue*); other South American countries (*light blue*), western Europe (*green*), eastern Europe (*light green*), North America (*purple*), Africa (*orange*), Middle East (*pink*), Asia (*grey*), Australia (*red*).

ancestor of the GP60 family containing repeat motives A16, G1, and R1, in which subsequently the TCA (16×) trinucleotide repeats (=A16) expanded in a stepwise manner to result in A24 repeats (Fig. 2, Supplementary Table S2). In this context, it must be noted that while a single nonsynonymous nucleotide mutation in a conserved region is a very rare singular event, microsatellite expansions belong to the most frequent type of genomic mutations observed (Li et al., 2002; Oliveira et al., 2006).

In addition to the above scenario, it must also be considered that worldwide livestock trade contributes to the global distribution of GP60 subtypes. To get an insight into the origin and worldwide distribution of the subtype family IIaAxxG1R1" (xx = 16–24) identified in Argentina, an exhaustive literature search was carried out. Surprisingly, we found that the greater the number of countries in which a subtype was identified, the lower the number of its "TCA" repeats (Fig. 2). Thus, the IIaA16G1R1 subtype is most widely distributed and was found in calves (n = 13), in humans (n = 4), and in calves and humans (n = 3) in altogether 20 different countries (Fig. 2, Supplementary Tables S1 and S2). In addition, when considering the allele subtype family IIaAxxG1R1 (xx = 16-24), this subtype has been reported to have the highest global prevalence of 6.4% (Chen et al., 2023). Both these findings strongly suggest that it represents the primordial subtype of this group of alleles. In contrast, the variant IIaA24G1R1, reported in only two countries from calves (n = 2), has been most likely generated much more recently. An exception from this finding was IIaA22G1R1 (n = 7), which shows a relatively wider geographical distribution than the subtype IIaA21G1R1 (n = 4) (Fig. 2, Supplementary Tables S1 and S2).

Further analysis of the distribution of the IIaAxxG1R1 (xx = 16–24) allele family in geographical world regions was carried out (Fig. 2). We found that all subtypes identified in Argentina and some neighbouring South American countries (Uruguay and Brazil) were predominantly identified from many countries of western and eastern Europe but also from other world regions such as Africa, North America, Middle East, and Asia. This distribution pattern is largely consistent with that of the currently known IIa subtypes as reported by Chen et al. (2023).

Recently, the GP60 subtypes IIaA15G2R1 (32.4%), IIaA18G3R1 (11.8%), and IIaA13G2R1 (8.2%) have been reported to be the globally most frequent (Chen et al., 2023). They display an increased copy number of the G repeat, which distinguishes them from the GP60 allele lineage IIaAxxG1R1. Interestingly subtypes with G extensions have not yet been identified in Argentina, which raises the question of how this finding may be interpreted. Since such consideration is related to the occurrence of random events such as genetic drift, it is by its very nature difficult to answer. However, it may be considered that an expansion of the A16 trinucleotide repeat should be at least 16 times more likely (e.g. by polymerase slippage) than that of the G nucleotide triplet since the A triplet is 16 times more frequent.

Interestingly, only variants IIaA16G1R1, IIaA17G1R1, IIaA18G1R1, and IIaA22G1R1 were reported in calves and humans within a given country, while the remaining subtypes were identified either in calves or humans (IIaA19G1R1 and IIaA20G1R1) or only in calves (IIaA21G1R1, IIaA23G1R1, and IIaA24G1R1) (Supplementary Tables S1 and S2). The zoonotic subtypes IIaA16G1R1, IIaA17G1R1, IIaA18G1R1, IIaA19G1R1, IIaA20G1R1, and IIaA22G1R1 identified in calves in Argentina represent 45.8% (77 out of 168) of the total of subtypes analysed, which suggests a high risk of human infection. Importantly, these subtypes have been also identified in human patients with diarrhoea in several countries around the world such as Australia, East Africa (Ethiopia), Europe (Great Britain, Ireland, Norway, Scotland, Spain, Sweden, UK), Middle East (Iraq), and North America (USA, Canada) (Fig. 2; Supplementary Table S1).

Noteworthy, subtypes IIaA16G1R1 and IIaA17G1R1 have been identified also by other authors in neonatal calves but were additionally found in the faecal material of sheep, pigs, dogs, lambs, horses and donkeys (Kvác et al., 2009; Smith et al., 2010; Imre et al., 2013; Ramo et al., 2014; Laatamna et al., 2015; Hijjawi et al., 2016; Kaupke et al., 2017; Rosanowski et al., 2018; Essid et al., 2018). The fact that they have been isolated from a wide variety of domestic animal species suggests that these may represent important reservoir hosts and a source of oocysts, which may consequently re-infect neonatal calves. Both subtypes are considered to be of zoonotic importance and were isolated on several occasions from diarrhoeic human patients (Thompson et al.,

2008). The reason for the ability of these particular *C. parvum* subtypes to infect various animal species requires further investigation and may indicate the presence of yet unrecognized species or a cryptic species complex.

6. Conclusions

Cryptosporidiosis of calves constitutes an economic burden for the cattle industry and is a concern for public health. The aim of this review was to analyse research data from Argentina to allow their comparison and integration with those reported from other countries. A high prevalence of dairy calf cryptosporidiosis of 25.2–42.5% in the age group of $\leq$ 20 days, and a prevalence of 16.3–25.5% in the age group of under 90 days was observed in the main dairy regions. These prevalence rates are significant, placing Argentina in the upper middle range of surveyed countries. In all cases where species identification was performed (n = 393), only *C. parvum* was identified in calves less than 90 days of age. Comparisons of different enteropathogens showed that the main risk factor for calf diarrhoea was the infection with *C. parvum*, while RVA was found to be of secondary importance. Other risk factors for *C. parvum* infections were a calf age under 20 days, a significantly increased prevalence of calf cryptosporidiosis in areas with poorly drained soils, a large size of herds (> 300 cattle heads), and co-infection of *C. parvum* with RVA; though mixed infections are rare and do therefore not account for an increased prevalence of calf diarrhoea. Surprisingly, the seamless identification of GP60 subtypes from IIaA16G1R1 to IIaA24G1R1 suggests a stepwise expansion of the trinucleotide repeat A16 to A24 within this region. This is supported by the presence of a non-synonymous nucleotide exchange in the conserved region of all identified GP60 subtypes. This notion is furthermore supported by our finding that the lower the repeat number (A16) the wider the worldwide geographical distribution of the respective GP60 subtype. Finally, nearly half of the subtypes identified in calves in Argentina (77 out of 168, corresponding to 45.8%) represented subtypes known to be zoonotic (IIaA16G1R1 to IIaA20G1R1, and IIaA22G1R1), suggesting a high risk of human infection. Our findings contribute to a deeper understanding and a more profound insight of calf cryptosporidiosis in Argentina and other regions worldwide.

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Not applicable.

CRediT authorship contribution statement

Paloma de Alba: Investigation, Formal analysis, Visualization, Writing – original draft. **Carlos Garro:** Writing – review & editing, Funding acquisition. **Monica Florin-Christensen:** Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Leonhard Schnittger:** Conceptualization, Formal analysis, Investigation, Methodology, Writing – review & editing, Visualization, Supervision, Funding acquisition, All authors read and approved the final manuscript.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data supporting the conclusions of this article are included within the article and its supplementary files.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2023.100147.

References

Aguirre, F., Ruiz, M.F., Allassia, M., Bagattin, L., Otero, J.L., 2014. Presencia de *Cryptosporidium* spp. en terneros de establecimientos lecheros de la provincia de Santa Fe (Argentinia). FAVE Sección Ciencias Veterinarias 13, 28–37. https://doi.org/10.14409/favecv.v13i1/2.4973.

Al Mawly, J., Grinberg, A., Prattley, D., Moffat, J., Marshall, J., French, N., 2015. Risk factors for neonatal calf diarrhoea and enteropathogen shedding in New Zealand dairy farms. Vet. J. 203, 155–160. https://doi.org/10.1016/j.tvjl.2015.01.010.

Aldeyarbi, H.M., Karanis, P., 2016. The fine structure of sexual stage development and sporogony of *Cryptosporidium parvum* in cell-free culture. Parasitology 143, 749–761. https://doi.org/10.1017/S0031182016000275.

Amer, S., Zidan, S., Adamu, H., Ye, J., Roellig, D., Xiao, L., Feng, Y., 2013. Prevalence and characterization of *Cryptosporidium* spp. in dairy cattle in Nile River Delta provinces, Egypt. Exp. Parasitol. 135, 518–523. https://doi.org/10.1016/j.exppara.2013.09.002.

Bartels, C.J., Holzhauer, M., Jorritsma, R., Swart, W.A., Lam, T.J., 2010. Prevalence, prediction and risk factors of enteropathogens in normal and non-normal faeces of young Dutch dairy calves. Prev. Vet. Med. 93, 162–169. https://doi.org/10.1016/j.prevetmed.2009.09.020.

Bellinzoni, R.C., Blackhall, J., Terzolo, H.R., Moreira, A.R., Auza, N., Mattion, N., et al., 1990. Microbiology of diarrhoea in young beef and dairy calves in Argentina. Rev. Argent. Microbiol. 22, 130–136.

Bertoni, E., Barragán, A.A., Bok, M., Vega, C., Martínez, M., Gil, J.F., et al., 2021. Assessment of influential factors for scours associated with *Cryptosporidium* sp., rotavirus and coronavirus in calves from Argentinean dairy farms. Animals 11, 2652. https://doi.org/10.3390/ani11092652.

Bouzid, M., Hunter, P.R., Chalmers, R.M., Tyler, K.M., 2013. *Cryptosporidium* pathogenicity and virulence. Clin. Microbiol. Rev. 26, 115–134. https://doi.org/10.1128/CMR.00076-12.

Bronsdon, M.A., 1984. Rapid dimethyl sulfoxide-modified acid-fast stain of *Cryptosporidium* oocysts in stool specimens. J. Clin. Microbiol. 19, 952–953.

Brook, E., Hart, C.A., French, N., Christley, R., 2008. Prevalence and risk factors for *Cryptosporidium* spp. infection in young calves. Vet. Parasitol. 152, 46–52. https://doi.org/10.1016/j.vetpar.2007.12.003.

Budu-Amoako, E., Greenwood, S.J., Dixon, B.R., Barkema, H.W., McClure, J.T., 2012. *Giardia* and *Cryptosporidium* on dairy farms and the role these farms may play in contaminating water sources in Prince Edward Island. Canada J. Vet. Intern. Med. 26, 668–673. https://doi.org/10.1111/j.1939-1676.2012.00930.x.

Bukhari, Z., Smith, H.V., 1995. Effect of three concentration techniques on viability of *Cryptosporidium parvum* oocysts recovered from bovine feces. J. Clin. Microbiol. 33, 2592–2595.

Cardoso, J.M., Silveira, F.L., Araújo, A.J., De Carvalho, J.C., Kanamura, H.Y., 2008. Occurrence of *Cryptosporidium* spp. in a dairy cattle herd from Caçapava municipality, São Paulo State, Brazil. Rev. Bras. Parasitol. Vet. 17 (Suppl. 1), 239–242.

Casemore, D.P., Armstrong, M., Sands, R.L., 1985. Laboratory diagnosis of cryptosporidiosis. J. Clin. Pathol. 38, 1337–1341.

Castro-Hermida, J.A., González-Losada, Y.A., Ares-Mazás, E., 2002. Prevalence of and risk factors involved in the spread of neonatal bovine cryptosporidiosis in Galicia (NW Spain). Vet. Parasitol. 106, 1–10. https://doi.org/10.1016/s0304-4017(02)00036-5.

Cerezuela, F., Miniti, E., Ocampo, M., Loustanau, M., Barrera, E., Ojeda, P., et al., 2017. *Cryptosporidium* sp. en la Provincia de La Rioja, Argentina. Niños asintomático con baja talla y coinfectados con *Giardia* sp. importancia del medio ambiente. Arch. Med. 13, 9.

Chalmers, R.M., Katzer, F., 2013. Looking for *Cryptosporidium*: the application of advances in detection and diagnosis. Trends Parasitol. 29, 237–251. https://doi.org/10.1016/j.pt.2013.03.001.

Checkley, W., White Jr., A.C., Jaganath, D., Arrowood, M.J., Chalmers, R.M., et al., 2015. A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for *Cryptosporidium*. Lancet Infect. Dis. 15, 85–94. https://doi.org/10.1016/S1473-3099(14)70772-8.

Chen, Y., Huang, J., Qin, H., Wang, L., Li, J., Zhang, L., 2023. *Cryptosporidium parvum* and gp60 genotype prevalence in dairy calves worldwide: A systematic review and meta-

analysis. Acta Trop. 240, 106843 https://doi.org/10.1016/j.actatropica.2023.106843.
Cho, Y.I., Yoon, K.J., 2014. An overview of calf diarrhea - infectious etiology, diagnosis, and intervention. J. Vet. Sci. 15, 1–17. https://doi.org/10.4142/jvs.2014.15.1.1.
de la Fuente, R., Luzón, M., Ruiz-Santa-Quiteria, J.A., García, A., Cid, D., Orden, J.A., et al., 1999. *Cryptosporidium* and concurrent infections with other major enterophatogens in 1 to 30-day-old diarrheic dairy calves in central Spain. Vet. Parasitol. 80, 179–185. https://doi.org/10.1016/s0304-4017(98)00218-0.
Deksne, G., Krūmiņš, A., Mateusa, M., Morozovs, V., Šveisberga, D.P., Korotinska, R., et al., 2022. Occurrence of *Cryptosporidium* spp. and *Giardia* spp. infection in humans in Latvia: Evidence of underdiagnosed and underreported cases. Medicina 58, 471. https://doi.org/10.3390/medicina58040471.
Del Coco, V.F., Córdoba, M.A., Basualdo, J.A., 2008. *Cryptosporidium* infection in calves from a rural area of Buenos Aires, Argentina. Vet. Parasitol. 158, 31–35. https://doi.org/10.1016/j.vetpar.2008.08.018.
Del Coco, V.F., Córdoba, M.A., Bilbao, G., de Almeida Castro, A.P., Basualdo, J.A., Fayer, R., Santín, M., 2014. *Cryptosporidium parvum* GP60 subtypes in dairy cattle from Buenos Aires, Argentina. Res. Vet. Sci. 96, 311–314. https://doi.org/10.1016/j.rvsc.2013.12.010.
Díaz, P., Hadfield, S.J., Quílez, J., Soilán, M., López, C., Panadero, R., et al., 2012. Assessment of three methods for multilocus fragment typing of *Cryptosporidium parvum* from domestic ruminants in North-West Spain. Vet. Parasitol. 186, 188–195. https://doi.org/10.1016/j.vetpar.2011.11.039.
Elsafi, S.H., Al-Sheban, S.S., Al-Jubran, K.M., Abu Hassan, M.M., Al Zahrani, E.M., 2014. Comparison of Kinyoun's acid-fast and immunofluorescent methods detected an unprecedented occurrence of *Cryptosporidium* in the eastern region of Saudi Arabia. J. Taibah Univ. Med. Sci. 9, 263–267.
Essid, R., Menotti, J., Hanen, C., Aoun, K., Bouratbine, A., 2018. Genetic diversity of *Cryptosporidium* isolates from human populations in an urban area of northern Tunisia. Infect. Genet. Evol. 58, 237–242. https://doi.org/10.1016/j.meegid.2018.01.004.
Fayer, R., Trout, J.M., Jenkins, M.C., 1998. Infectivity of *Cryptosporidium parvum* oocysts stored in water at environmental temperatures. J. Parasitol. 84, 1165–1169.
Fayer, R., 2010. Taxonomy and species delimitation in *Cryptosporidium*. Exp. Parasitol. 124, 90–97. https://doi.org/10.1016/j.exppara.2009.03.005.
Feng, Y., Ortega, Y., He, G., Das, P., Xu, M., Zhang, X., et al., 2007. Wide geographic distribution of *Cryptosporidium bovis* and the deer-like genotype in bovines. Vet. Parasitol. 144, 1–9. https://doi.org/10.1016/j.vetpar.2006.10.001.
Florin-Christensen, M., Schnittger, L., Bastos, R.G., Vignesh, A.R., Cooke, B.M., Alzan, H. F., Suarez, C.E., 2021. Pursuing effective vaccines against cattle diseases caused by apicomplexan protozoa. CAB Reviews 16, 1–23. https://doi.org/10.1079/PAVSNNR202116024.
Follet, J., Guyot, K., Leruste, H., Follet-Dumoulin, A., Hammouma-Ghelboun, O., Certad, G., et al., 2011. *Cryptosporidium* infection in a veal calf cohort in France: Molecular characterization of species in a longitudinal study. Vet. Res. 42, 116. https://doi.org/10.1186/1297-9716-42-116.
Foster, D.M., Smith, G.W., 2009. Pathophysiology of diarrhea in calves. Vet. Clin. North Am. Food Anim. Pract. 25, 13–36. https://doi.org/10.1016/j.cvfa.2008.10.013.
Garcia, L.S., 2007. Diagnostic Medical Parasitology. American Society for Microbiology Press, Washington DC.
Garro, C.J., Morici, G.E., Tomazic, M.L., Vilte, D., Encinas, M., Vega, C., et al., 2021. Occurrence of *Cryptosporidium* and other enteropathogens and their association with diarrhea in dairy calves of Buenos Aires Province, Argentina, Vet. Parasitol. Reg. Stud. Rep. 24, 100567 https://doi.org/10.1016/j.vprsr.2021.100567.
Garro, C.J., Morici, G.E., Utgés, M.E., Tomazic, M.L., Schnittger, L., 2016. Prevalence and risk factors for shedding of *Cryptosporidium* spp. oocysts in dairy calves of Buenos Aires Province, Argentina. Parasite Epidemiol. Control 1, 36–41. https://doi.org/10.1016/j.parepi.2016.03.008.
Gerace, E., Lo Presti, V.D.M., Biondo, C., 2019. *Cryptosporidium* infection: Epidemiology, pathogenesis, and differential diagnosis. Eur. J. Microbiol. Immunol. 9, 119–123. https://doi.org/10.1556/1886.2019.00019.
Guo, Y., Ryan, U., Feng, Y., Xiao, L., 2022. Association of common zoonotic pathogens with concentrated animal feeding operations. Front. Microbiol. 10 (12), 810142 https://doi.org/10.3389/fmicb.2021.810142.
Guy, R.A., Yanta, C.A., Muchaal, P.K., Rankin, M.A., Thivierge, K., Lau, R., 2021. Molecular characterization of *Cryptosporidium* isolates from humans in Ontario, Canada. Parasites Vectors 14, 69. https://doi.org/10.1186/s13071-020-04546-9.
Henriksen, S.A., Pohlenz, J.F.L., 1981. Staining of cryptosporidia by a modified Ziehl-Neelsen technique. Acta Vet. Scand. 22, 594–596.
Hijjawi, N., Mukbel, R., Yang, R., Ryan, U., 2016. Genetic characterization of *Cryptosporidium* in animal and human isolates from Jordan. Vet. Parasitol. 228, 116–120. https://doi.org/10.1016/j.vetpar.2016.08.015.
Hur, T.Y., Jung, Y.H., Choe, C.Y., Cho, Y.I., Kang, S.J., Lee, H.J., et al., 2013. The dairy calf mortality: The causes of calf death during ten years at a large dairy farm in Korea. Korean J. Vet. Res. 53, 103–108. https://doi.org/10.14405/kjvr.2013.53.2.103.
Imre, K., Luca, C., Costache, M., Sala, C., Morar, A., Morariu, S., et al., 2013. Zoonotic *Cryptosporidium parvum* in Romanian newborn lambs (*Ovis aries*). Vet. Parasitol. 191, 119–122. https://doi.org/10.1016/j.vetpar.2012.08.020.
Izzo, M.M., Kirkland, P.D., Mohler, V.L., Perkins, N.R., Gunn, A.A., House, J.K., 2011. Prevalence of major enteric pathogens in Australian dairy calves with diarrhoea. Austral. Vet. J. 89, 167–173. https://doi.org/10.1111/j.1751-0813.2011.00692.x.
Jenkins, M.B., Eaglesham, B.S., Anthony, L.C., Kachlany, S.C., Bowman, D.D., Ghiorse, W.C., 2010. Significance of wall structure, macromolecular composition, and surface polymers to the survival and transport of *Cryptosporidium parvum* oocysts. Appl. Environ. Microbiol. 76, 1926–1934. https://doi.org/10.1128/AEM.02295-09.
Kaupke, A., Michalski, M.M., Rzeżutka, A., 2017. Diversity of *Cryptosporidium* species occurring in sheep and goat breeds reared in Poland. Parasitol. Res. 116, 871–879. https://doi.org/10.1007/s00436-016-5360-3.
Khanna, V., Tilak, K., Ghosh, A., Mukhopadhyay, C., 2014. Modified negative staining of Heine for fast and inexpensive screening of *Cryptosporidium*, *Cyclospora*, and *Cystoisospora* spp. Int. Sch. Res. Notices 2014, 165424. https://doi.org/10.1155/2014/165424.
Kvác, M., Hanzlíková, D., Sak, B., Kvetonová, D., 2009. Prevalence and age-related infection of *Cryptosporidium suis*, *C. muris* and *Cryptosporidium* pig genotype II in pigs on a farm complex in the Czech Republic. Vet. Parasitol. 160, 319–322. https://doi.org/10.1016/j.vetpar.2008.11.007.
Laatamna, A.E., Wagnerová, P., Sak, B., Květoňová, D., Xiao, L., Rost, M., et al., 2015. Microsporidia and *Cryptosporidium* in horses and donkeys in Algeria: Detection of a novel *Cryptosporidium hominis* subtype family (Ik) in a horse. Vet. Parasitol. 208, 135–142. https://doi.org/10.1016/j.vetpar.2015.01.007.
Li, Y.-C., Korol, A.B., Fahima, T., Beiles, A., Nevo, E., 2002. Microsatellites: Genomic distribution, putative functions and mutational mechanisms: a review. Mol. Ecol. 11, 2453–2465. https://doi.org/10.1046/j.1365-294X.2002.01643.x.
Lombardelli, J.A., Tomazic, M.L., Schnittger, L., Tiranti, K.I., 2019. Prevalence of *Cryptosporidium parvum* in dairy calves and GP60 subtyping of diarrheic calves in central Argentina. Parasitol. Res. 118, 2079–2086. https://doi.org/10.1007/s00436-019-06366-y.
Maddox-Hyttel, C., Langkjaer, R.B., Enemark, H.L., Vigre, H., 2006. *Cryptosporidium* and *Giardia* in different age groups of Danish cattle and pigs - occurrence and management associated risk factors. Vet. Parasitol. 141, 48–59. https://doi.org/10.1016/j.vetpar.2006.04.032.
Maldonado-Camargo, S., Atwill, E.R., Saltijeral-Oaxaca, J.A., Herrera-Alonso, L.C., 1998. Prevalence of and risk factors for shedding of *Cryptosporidium parvum* in Holstein Freisian dairy calves in central México. Prev. Vet. Med. 36, 95–107. https://doi.org/10.1016/s0167-5877(98)00084-1.
Meireles, M.V., de Oliveira, F.P., Teixeira, W.F., Coelho, W.M., Mendes, L.C., 2011. Molecular characterization of *Cryptosporidium* spp. in dairy calves from the state of São Paulo, Brazil. Parasitol. Res. 109, 949–951. https://doi.org/10.1007/s00436-011-2336-1.
Messner, M.J., Berger, P., 2016. *Cryptosporidium* infection risk: Results of new dose-response modeling. Risk Anal. 36, 1969–1982. https://doi.org/10.1111/risa.12541.
Modini, L., Otero, J.L., Carrera, E., Zerbatto, M., Eliggi, S., Abramovich, B., 2010. *Cryptosporidium* spp. en ganado bovino: Su potential como contaminante de los recursos hídricos. FAVE Sección Ciencias Veterinarias 9, 33–38. https://doi.org/10.14409/favecv.v9i1.1494.
Modini, L.B., Carrera, E., Otero, J.L., Zerbatto, M.G., Eliggi, M.S., Vaira, S., Abramovich, B.L., 2011. Infección por *Cryptosporidium* spp. en ganado vacuno de la cuenca lechera de la provincia de Santa Fe (Argentina). FABICIB 15, 97–107. https://doi.org/10.14409/fabicib.v15i1.884.
Nydam, D.V., Wade, S.E., Schaaf, S.L., Mohammed, H.O., 2001. Number of *Cryptosporidium parvum* oocysts or *Giardia* spp. cysts shed by dairy calves after natural infection. Am. J. Vet. Res. 62, 1612–1615. https://doi.org/10.2460/ajvr.2001.62.1612.
Oliveira, E.J., Pádua, J.G., Zucchi, M.I., Vencovsky, R., Vieira, M.L., 2006. Origin, evolution and genome distribution of microsatellites. Genet. Mol. Biol. 29, 294–307.
Petry, F., 2004. Structural analysis of *Cryptosporidium parvum*. Microsc. Microanal. 10, 586–601. https://doi.org/10.1017/S1431927604040929.
Potters, I., Van Esbroeck, M., 2010. Negative staining technique of Heine for the detection of *Cryptosporidium* spp: A fast and simple screening technique. Parasitol. Open 4, 1–4.
Ramo, A., Quílez, J., Del Cacho, E., Sánchez-Acedo, C., 2014. Optimization of a fragment size analysis tool for identification of *Cryptosporidium* species and GP60 alleles infecting domestic ruminants. Vet. Parasitol. 205, 466–471. https://doi.org/10.1016/j.vetpar.2014.08.025.
Rosanowski, S.M., Banica, M., Ellis, E., Farrow, E., Harwood, C., Jordan, B., et al., 2018. The molecular characterisation of *Cryptosporidium* species in relinquished dogs in Great Britain: A novel zoonotic risk? Parasitol. Res. 117, 1663–1667. https://doi.org/10.1007/s00436-018-5857-z.
Sanford, S.E., Josephson, G.K., 1982. Bovine cryptosporidiosis: Clinical and pathological findings in forty-two scouring neonatal calves. Can. Vet. J. 23, 343–347.
Santín, M., Trout, J.M., Fayer, R., 2008. A longitudinal study of cryptosporidiosis in dairy cattle from birth to 2 years of age. Vet. Parasitol. 155, 15–23. https://doi.org/10.1016/j.vetpar.2008.04.018.
Santin, M., 2020. *Cryptosporidium* and *Giardia* in ruminants. Vet. Clin. North Am. Food Anim. Pract. 36, 223–238. https://doi.org/10.1016/j.cvfa.2019.11.005.
Silverlås, C., Näslund, K., Björkman, C., Mattsson, J.G., 2010. Molecular characterisation of *Cryptosporidium* isolates from Swedish dairy cattle in relation to age, diarrhoea and region. Vet. Parasitol. 169, 289–295. https://doi.org/10.1016/j.vetpar.2010.01.003.
Smith, R.P., Chalmers, R.M., Mueller-Doblies, D., Clifton-Hadley, F.A., Elwin, K., Watkins, J., et al., 2010. Investigation of farms linked to human patients with cryptosporidiosis in England and Wales. Prev. Vet. Med. 94, 9–17. https://doi.org/10.1016/j.prevetmed.2009.12.005.
Sow, S.O., Muhsen, K., Nasrin, D., Blackwelder, W.C., Wu, Y., Farag, T.H., et al., 2016. The burden of *Cryptosporidium* diarrheal disease among children < 24 months of age in moderate/high mortality regions of sub-Saharan Africa and South Asia, utilizing data from the Global Enteric Multicenter Study (GEMS). PLoS Negl. Trop. Dis. 10, e0004729.

Thompson, R.A., Fayer, R., Xiao, L., 2008. *Cryptosporidium* and cryptosporidiosis. Parasites Vectors 1, 47. https://doi.org/10.1186/1756-3305-1-47.

Thomson, S., Innesi, E.A., Jonsson, N.N., Katzer, F., 2016. A multiplex PCR test to identify four common cattle-adapted *Cryptosporidium* species. Parasitol. Open 2, 1–9. https://doi.org/10.1017/pao.2016.2.

Tiranti, K., Larriestra, A., Vissio, C., Picco, N., Alustiza, F., Degioanni, A., Vivas, A., 2011. Prevalence of *Cryptosporidium* spp. and *Giardia* spp., spatial clustering and patterns of shedding in dairy calves from Córdoba, Argentina. Rev. Bras. Parasitol. Vet. 20, 140–147. https://doi.org/10.1590/s1984-29612011000200009.

Toledo, R.D., Martins, F.D., Ferreira, F.P., de Almeida, J.C., Ogawa, L., Dos Santos, H.L., et al., 2017. *Cryptosporidium* spp. and *Giardia* spp. in feces and water and the associated exposure factors on dairy farms. PLoS One 12, e0175311. https://doi.org/10.1371/journal.pone.0175311.

Tomazic, M.L., Maidana, J., Dominguez, M., Uriarte, E.L., Galarza, R., Garro, C., et al., 2013. Molecular characterization of *Cryptosporidium* isolates from calves in Argentina. Vet. Parasitol. 198, 382–386. https://doi.org/10.1016/j.vetpar.2013.09.022.

Tomazic, M.L., Garro, C.J., Schnittger, L., 2018. *Cryptosporidium*. In: Florin-Christensen, M., Schnittger, L. (Eds.), Parasitic Protozoa of Farm Animals and Pets. Springer International Publishing, NY, pp. 11–54.

Trotz-Williams, L.A., Wayne Martin, S., Leslie, K.E., Duffield, T., Nydam, D.V., Peregrine, A.S., 2007. Calf-level risk factors for neonatal diarrhea and shedding of *Cryptosporidium parvum* in Ontario dairy calves. Prev. Vet. Med. 82, 12–28. https://doi.org/10.1016/j.prevetmed.2007.05.003.

USDA, 2008. Dairy 2007 Part II: Changes in the U.S. Dairy Cattle Industry 1991–2007. USDA-APHIS-VS, CEAH, Fort Collins, pp. 57–61. www.aphis.usda.gov/animal_health/nahms/dairy/downloads/dairy07/Dairy07_is_PartII_Highlights_1.pdf.

Vermeulen, L.C., Benders, J., Medema, G., Hofstra, N., 2017. Global *Cryptosporidium* loads from livestock manure. Environ. Sci. Technol. 51, 8663–8671. https://doi.org/10.1021/acs.est.7b00452.

Wang, R., Zhao, R., Gong, Y., Zhang, L., 2017. Advances and perspectives on the epidemiology of bovine *Cryptosporidium* in China in the past 30 years. Front. Microbiol. 8, 1823. https://doi.org/10.3389/fmicb.2017.01823.

Wade, S.E., Mohammed, H.O., Schaaf, S.L., 2000. Prevalence of *Giardia* sp. *Cryptosporidium parvum* and *Cryptosporidium andersoni* (syn. *C. muris*) in 109 dairy herds in five counties of southeastern New York. Vet. Parasitol. 93, 1–11. https://doi.org/10.1016/s0304-4017(00)00337-x.

Wang, L., Wang, Y., Cui, Z., Li, D., Li, X., Zhang, S., Zhang, L., 2022. Enrichment and proteomic identification of *Cryptosporidium parvum* oocyst wall. Parasites Vectors 15, 335. https://doi.org/10.1186/s13071-022-05448-8.

Widmer, G., Feng, X., Tanriverdi, S., 2004. Genotyping of *Cryptosporidium parvum* with microsatellite markers. Methods Mol. Biol. 268, 177–187. https://doi.org/10.1385/1-59259-766-1:177.

Wyatt, C.R., Riggs, M.W., Fayer, R., 2010. Cryptosporidiosis in neonatal calves. Vet. Clin. North Am. Food Anim. Pract. 26 https://doi.org/10.1016/j.cvfa.2009.10.001.

Xiao, L., Escalante, L., Yang, C., Escalante, A.A., Montali, R.J., Fayer, R., et al., 1999. Phylogenetic analysis of *Cryptosporidium* parasites based on the small subunit rRNA gene locus. Appl. Environ. Microbiol. 65, 1578–1583. https://doi.org/10.1128/AEM.65.4.1578-1583.1999.

Xiao, L., Feng, Y., 2017. Molecular epidemiologic tools for waterborne pathogens *Cryptosporidium* spp. and *Giardia duodenalis*. Food Waterborne Parasitol 8–9, 14–32. https://doi.org/10.1016/j.fawpar.2017.09.002.

Xiao, L., 2010. Molecular epidemiology of cryptosporidiosis: An update. Exp. Parasitol. 124, 80–89. https://doi.org/10.1016/j.exppara.2009.03.018.

Xiao, L., Feng, Y., 2008. Zoonotic cryptosporidiosis. FEMS Immunol. Med. Microbiol. 52, 309–323. https://doi.org/10.1111/j.1574-695X.2008.00377.x.

Xiao, L., Zhou, L., Santin, M., Yang, W., Fayer, R., 2007. Distribution of *Cryptosporidium parvum* subtypes in calves in eastern United States. Parasitol. Res. 100, 701–706. https://doi.org/10.1007/s00436-006-0337-2.

Yang, X., Guo, Y., Xiao, L., Feng, Y., 2021. Molecular epidemiology of human cryptosporidiosis in low- and middle-income countries. Clin. Microbiol. Rev. 34, e00087-19 https://doi.org/10.1128/CMR.00087-19.

Young, K.H., Bullock, S.L., Melvin, D.M., Spruill, C.L., 1979. Ethyl acetate as a substitute for diethyl ether in the formalin ether sedimentation technique. J. Clin. Microbiol. 10, 852–885.

Zambriski, J.A., Nydam, D.V., Wilcox, Z.J., Bowman, D.D., Mohammed, H.O., Liotta, J. L., 2013. *Cryptosporidium parvum*: Determination of ID_{50} and the dose-response relationship in experimentally challenged dairy calves. Vet. Parasitol. 197, 104–112. https://doi.org/10.1016/j.vetpar.2013.04.022.