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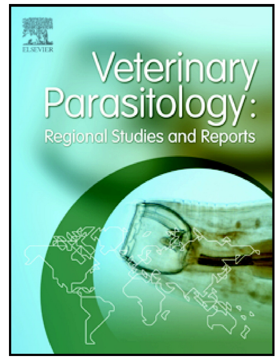

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PII: S2405-9390(21)00039-3

DOI: https://doi.org/10.1016/j.vprsr.2021.100567

Reference: VPRSR 100567

To appear in: *Veterinary Parasitology: Regional Studies and Reports*

Received date: 9 December 2020

Revised date: 15 March 2021

Accepted date: 30 March 2021

Please cite this article as: C.J. Garro, G.E. Morici, M.L. Tomazic, et al., Occurrence of Cryptosporidium and other enteropathogens and their association with diarrhea in dairy calves of Buenos Aires province, Argentina, *Veterinary Parasitology: Regional Studies and Reports* (2021), https://doi.org/10.1016/j.vprsr.2021.100567

Occurrence of *Cryptosporidium* and other enteropathogens and their association with diarrhea in dairy calves of Buenos Aires province, Argentina

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Abstract

Cryptosporidiosis of neonatal dairy calves causes diarrhea, resulting in important economic losses. In Argentina, prevalence values of *Cryptosporidium* spp. and other enteropathogens such as group A rotavirus (RVA), bovine coronavirus (BCoV) and enterotoxigenic *Escherichia coli* (ETEC, endotoxin STa^+), have been independently studied in different regions. However, an integrative epidemiological investigation on large-scale farms has not been carried out. In this study, fecal samples (*n* = 908) were randomly collected from diarrheic and healthy calves from 42 dairy farms, and analyzed for the presence of *Cryptosporidium* spp., RVA, BCoV, ETEC (STa^+) and *Salmonella* spp. In all sampled dairy farms, dams had been vaccinated against rotavirus and gram-negative bacteria to protect calves against neonatal diarrhea. The proportion of calves shedding *Cryptosporidium* spp., RVA, and BCoV in animals younger than 20 days of age were 29.8 %, 12.4 % and 6.4 % , and in calves aged between 21 and 90 days, 5.6 %, 3.9 %, and 1.8 %, respectively. ETEC was absent in the younger, and occurred only sporadically in the older group (0.9%), whereas *Salmonella* spp. was absent in both. The observed sporadic finding or even absence of bacterial pathogens might be explained by the frequent use of parenteral antibiotics in 25.3% and 6.5% of the younger and the older group of calves, respectively, within 2 days prior to sampling and/or vaccination of dams against gram-negative bacteria. Diarrhea was observed in 28.8% (95% CI, 24.7–32.8%) of the younger calves and

11.7% (95% CI, 9.1–15.5%) of the older calves. Importantly, *Cryptosporidium* spp. (odds ratio (OR) = 5.7; 95% CI, 3.3–9.9; $p<0.0001$) and RVA (OR= 2.5; 95% CI, 1.2–5.1; $p<0.05$) were both found to be risk factors for diarrhea in calves younger than 20 days old. Based on its high prevalence and OR, our results strongly suggest that *Cryptosporidium* spp. is the principal causative factor for diarrhea in the group of neonatal calves, whereas RVA seems to play a secondary role in the etiology of diarrhea in the studied farms, with about three-times lower prevalence and a half as high OR. Furthermore, a coinfection rate of *Cryptosporidium* spp. and RVA of 3.7% was observed in the group of younger calves, which strengthens the assumption that these events are independent. In contrast, due to a low infection rate of enteropathogens in older calves, mixed infection (<< 1%) was virtually absent in this group.

Keywords: *Cryptosporidium* spp.; *Cryptosporidium parvum;* rotavirus; enteropathogens; calf diarrhea; odds ratio.

1. Introduction

Diarrhea of neonatal calves is a major sanitary problem in dairy farms worldwide (Olsen et al., 2004). It has been reported that heifers that suffered from neonatal diarrhea, conceived and calved at a later age, decreasing reproductive performance (Aghakeshmiri et al., 2017). Furthermore, heifers with a history of diarrhea showed, on one hand, a reduced gain of live weight resulting in a poorer production performance and, on the other hand, a substantially reduced milk production during the first lactation period (Svensson and Hultgren, 2008; Bueno da Silva et al., 2019). The etiology of diarrhea is complex and may be caused by single or multiple parasitic, bacterial, and virus infections, and is also facilitated by a range of nutritional, environmental and/or management conditions (Cho and Yoon, 2014). During a diarrhea episode of a new born calf, loss of body fluids and essential electrolytes may eventually lead to dehydration and metabolic distress of the animal, resulting in significant direct economic losses due to therapeutic costs and possible animal death, and/or indirect losses due to the associated weight reduction, which is not compensated after recovery (Millemann, 2009).

In Argentina, *C. parvum* has been reported to occur with a prevalence between 16 and 25.5% in dairy calves, and found to be strongly associated with diarrhea (Del Coco et al., 2008; Tiranti et al. 2011; Garro et al., 2016; Lombardelli et al. 2019). In other countries, such as New Zealand, Spain, USA, France, Iran and Canada, infection of neonatal calves with *C. parvum* varied within a considerable range from 16 to 52 %, and has been likewise found to be associated with diarrhea (de la Fuente et al., 1998; Trotz-Williams et al., 2005; Silverlås et al., 2009; Al Mawly et al., 2015b; Delafosse et al., 2015). Besides *C. parvum*, bovine group A rotavirus (RVA) is also considered to represent an important cause of neonatal calf diarrhea in Argentina and worldwide (Costantini et al., 2002; Garaicoechea et al., 2006; Badaracco et al., 2012). A prevalence of RVA in neonatal calves of 17.7% has been reported in farms of The Netherlands (< 21-day-old calves) (Bartels et al., 2010) and of 19.8% in New Zealand (9 to 21-day-old calves) (Al Mawly et al., 2015b). In contrast to RVA, bovine coronavirus (BCoV) shedding is commonly reported with a considerable lower prevalence. Accordingly, a low infection rate of 1.7% has been reported in diarrheic calves sampled from 1994 to 2010 in ten different provinces of Argentina (Bok et al., 2015). Enteropathogenic *E. coli* (ETEC) was found in dairy calves of younger age (< 10 days old) (Bartels et al. 2010; Cho et al. 2013; Al Mawly et al. 2015a; Picco et al. 2015; González Pasayo et al. 2019), whereas prevalence values of 5.9% (1 to 53-day-old calves) (Bilbao et al., 2019) and 4% (9 to 21-day-old calves) have been observed for *Salmonella* spp. (Al Mawly et al., 2015a).

Notably, independent studies have demonstrated the prevalence and importance of the enteropathogens *C. parvum* and RVA, and, to a lesser degree, of BCoV, *Escherichia coli* and *Salmonella*, in the etiology of diarrhea of dairy calves in Argentina

(Del Coco, 2008; Tiranti et al., 2011; Badaracco et al., 2012; Bok et al., 2015; Picco et al., 2015; Garro et al., 2016; Bilbao et al., 2019; Lombardelli et al., 2019). However, a study that demonstrates and compares the association of each agent with diarrhea in calves and/or detects the occurrence of possible synergetic effects of coinfections has not yet been carried out in Argentina. Accordingly, the aim of the present study was to estimate the frequency of *Cryptosporidium* shedding and its association with diarrhea in dairy calves of 1 to 20 and 21 to 90 days of age in large scale farms of the study region, and to compare it with those of viral and bacterial enteropathogens.

2. Materials and methods

2.1. Sampling frame and study design

A cross-sectional study was designed and sampling was carried out between august 2014 and august 2018. The target sample size of 908 dairy calves was determined based on an estimated 18% overall frequency of *Cryptosporidium* infection of calves (Del Coco et al., 2008; Tiranti et al., 2011), allowing for a maximum error of 2.5% and a confidence level of 95% (Dohoo et al., 2003).

With the assistance of private veterinarians and agronomists, dairy farms with more than 150 milking cows were selected by convenience of geographical proximity to the laboratory work place. All dairy farms included in our study were run under professional care and employed vaccination of dams against RVA, *E. coli*, and

Salmonella spp. in order to transfer passive immunity against neonatal diarrhea to calves through colostrum. A minimum farm size of 150 milking cows was established to allow sampling of multiple calves in order to optimize estimation of the prevalence at the farm level. Up to 25 calves were sampled individually each time a dairy herd was visited. If less than 25 calves were available, all calves aged 1 to 90 days were sampled. If more than 25 calves were available, 15 calves under 30 days of age and 10 samples of older animals up to 90 days of age were collected. In a single case, 35 samples were collected in a dairy herd. The outlined sampling approach resulted finally in the collection of 908 fecal samples from a total of 42 dairy farms. All sampled calves were of the Holando-Argentino breed.

Feces were categorized at the time of collection by their fluidity as proposed by Larson et al. (1977). Correspondingly, liquid and semi-liquid feces were classified with a score of 2 and recorded as diarrheic, whereas firm or slightly deformed feces received a score of 1 and were recorded as non-diarrheic. In addition, the use of parenteral antibiotics in calves within 48 h prior to sampling was registered. A single fecal sample per calf was collected in a clean polyethylene bag directly from the rectum after anal massage or immediately after deposition. Samples were stored at 4°C until further processing in the laboratory within 24 h. Samples were aliquoted for subsequent analysis for the presence of *Cryptosporidium* spp./*C. parvum*, RVA, BCoV, ETEC, and *Salmonella* spp.

2.2. Cryptosporidium spp. and C. parvum identification

Fecal smears on slides were prepared, air dried, and fixed with methanol. Subsequently, Kinyoun staining was performed as previously described (Elsafi et al., 2014; Henriksen and Pohlenz, 1981), and preparations were examined by microscopy using oil immersion at 1000x magnification. Oocysts of *Cryptosporidium* were identified based on the morphology, optical properties, internal structure, and size, as described by Trotz-Williams et al. (2005) and Fayer et al. (1998). Up to 40 randomly selected fields were examined until the first detection of oocysts in a field rendered the sample positive, whereas the result was considered negative when no oocysts were observed in any of the examined 40 fields. For species identification, DNA was isolated from 200 mg of an oocyst-positive fecal sample as described in Tomazic et al. (2013), and purified DNA was quantified in a Nanodrop spectrophotometer and stored at −20 °C until further analysis. Subsequently, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for species determination was carried out, as described in Xiao et al. (1999) including the use of an additional restriction enzyme, *Mbo*II (Feng et al., 2007).

2.3. Viral identification

Fecal samples were screened for RVA using a VHH monoclonal capture ELISA (Garaicoechea et al., 2008). Briefly, 96-well plates (Maxisorp, NUNC, USA) were coated with VHH recombinant nanobodies against RVA at 4 °C overnight. Plates were then blocked using 10 % low-fat milk at room temperature during 45 min. One to ten dilutions of fecal samples in PBS were added and the plate was incubated at room temperature for 45 min. Finally, anti-RVA VHH nanobodies conjugated with soybean peroxidase were added and incubated for 45 min at room temperature. After incubation with tetramethylbenzidine colorimetric substrate (Life sciences, USA), absorbance was measured at a wavelength of 450 nm using an ELISA reader (Thermo scientific, USA). BCoV antigen detection in fecal samples was performed by an indirect antigen-capture ELISA as reported by Smith et al., (1996), with the modification described in Bok et al., (2015).

2.4. E. coli and Salmonella spp. identification

Fecal samples were cultured on MacConkey agar plates (Oxoid, Basingstoke,UK) and Gram staining was performed on lactose-positive colonies. Compatible colonies were biochemically characterized and transferred to tubes and cultured in LB broth for 8 h at 37 °C. After centrifugation, the bacterial pellet was subjected to DNA extraction. Since the STa enterotoxin is considered the principal diarrheagenic factor, all samples found to be PCR positive for the corresponding encoding gene were considered to be positive for ETEC (Franck et al., 1998). To analyze the presence of *Salmonella* spp., samples were enriched in Rappaport-Vlassiliadis broth (Oxoid, Basingstoke, UK) for 48 h at 37 °C. Subsequently, samples were plated onto Xylose-Lysine-Deoxycholate agar

(Oxoid, Basingstoke, UK) and the presence of *Salmonella*-compatible colonies was biochemically confirmed (Caffarena et al., 2021).

2.5. Data Analysis

All data corresponding to farms and individual animals were recorded in an Access database (Microsoft Office Professional Plus©, 2016 Microsoft Corporation). In a previous study, calves with diarrhea predominantly belonged to the group aged less than 20 days (Garro et al., 2016; Millemann, 2009). For this reason, in the present study, animals were separated into two groups, aged 1 to 20 days and 21 to 90 days. Separate statistical analyses were conducted for each group. A two-sample proportion test was done to compare the frequency of enteropathogen shedding and diarrhea between the two age groups. The confidence interval of the frequency of diarrhea was calculated with WinEpi 2.0 (De Blas et al., 2006). Analysis of associations was based on a dichotomous outcome corresponding to calves with diarrhea *vs*. calves without diarrhea. Odds ratios (OR) and 95% confidence interval (CI) were considered statistically significant at $p<0.05$ and when not overlapping the null value. Bivariate and multivariate analyses were conducted with generalized logistic mixed models using the package "lme4" (Bates et al., 2015) for R© v. 3.0.2 (R Foundation for Statistical Computing), with logit link and fitted by maximum likelihood (Gauss–Hermite Quadrature). The "farm" variable (a unique identifier for each farm) was modelled as random effect. Model parameters were dropped using AIC (Akaike Information

Criterion): when AIC differed by 2 or more units, the simpler model was chosen. OR and their 95% CI were calculated. Variables found to be significant in the final multivariable model were assessed for interaction.

3. Results

3.1. Descriptive data

The amount of milking cows per sampled farm ranged from 150 to 2100, with a median of 315. The median of calves sampled per farm was 25 (minimum = 9; maximum = 35). A total of 42 dairy farms were included in this study situated in the districts of Alberti (*n*= 2), Carlos Casares (*n* = 1), Carmen de Areco (*n* = 2), Castelli (*n* = 1), Chacabuco (*n* = 3), Exaltación de la Cruz (*n* = 2), General Belgrano (*n* = 2), Lobos (*n* = 11), Lujan (*n* = 5), Marcos Paz (*n* = 3), Mercedes (*n* = 1), Monte (*n* = 3), Navarro (*n* = 3) and Suipacha (*n* = 3), all located in the northwestern region of Buenos Aires province, Argentina (Fig. 1). The sample represents 8.3% (42/503) of the total number of dairy herds in these districts. Furthermore, the 14 visited districts contain 19.1% (503/2626) of all dairy herds in the province of Buenos Aires (Ministry of Agricultural Affairs, 2009).

3.2. Frequency of enteropathogens

Fifty-nine of 168 calves (35.1%) that tested positive for oocysts of *Cryptosporidium* spp. by Ziehl-Neelsen staining could be determined as *C. parvum* by PCR-RFLP. With the exception of a single calf, all oocyst-positive calves were under 60 days of age (Supplementary Table 1). Among all calves younger than 20 days of age, the proportions shedding *Cryptosporidium* spp., RVA, BCoV and ETEC were of 29.8, 12.4, 6.4 and 0%, respectively, whereas among 21 to 90-day-old calves, they were of 5.6, 3.9, 1.8, and 0.9%, respectively.

No shedding of *Salmonella* spp. was observed in any of the examined animals. The frequencies of enteropathogen occurrence at the calf and herd level according to their clinical condition (with or without diarrhea) and the frequencies of co-infections with two or more enteropathogens are shown in Table 1. The frequency of enteropathogen infections of calves observed at different weeks of age was highest for *Cryptosporidium* spp. at week two (38.8%) and three (28%), for RVA at week one (13.9%) and two (15%), and for coronavirus at week one (12.4%). In contrast, ETEC was only found sporadically between week six and ten (Supplementary Table 1).

3.3. Frequency of diarrhea and associated enteropathogens

The frequency of diarrhea was significantly higher in 1 to 20-day-old calves (28.8%; 95%CI: 24.7–32.8%) as compared to those aged 21 to 90 days (11.7%; 95%CI: 9.1–15.5%) (Proportion test, $p<0.001$). The frequency of use of parenteral antibiotics within 48 h prior to sampling was 25.3% and 6.5% in the 1 to 20 and the 21 to 90-day-old groups, respectively.

In the 1 to 20-day-old group, bivariate screening identified three variables that were independently associated with increased odds of diarrhea: shedding of *Cryptosporidium* spp., shedding of RVA and concurrent shedding of *Cryptosporidium* spp and RVA (Table 2). However, the most parsimonious model included only RVA and *Cryptosporidium* spp. infections with random effect of farm. In Table 3, the final model shows that the odds of diarrhea are highly significantly increased when there is shedding of *Cryptosporidium* spp. (OR= 5.7; 95% CI= 3.3– 9.9; p<0.0001) and significantly increased for shedding of RVA (OR= 2.5; 95% CI= 1.2– 5.1; p=0.010).

In the 21 to 90-day-old group, none of the enteropathogens identified and analyzed in the context of the study was found to be associated with presence of diarrhea. Furthermore, since the rate of infection of none of the studied enteropathogens exceeded 10 %, the frequency of mixed infections was extremely low (<< 1%).

4. Discussion

The proportion of calves shedding *Cryptosporidium* and bacterial and viral enteropathogens was determined in a cross-sectional study that included 42 dairy farms with a herd size of more than 150 milking cows in the northwestern region of Buenos Aires province, Argentina. The farms of this appraisal comprised 8.3% of all dairy farms in the study region.

In three previous studies, altogether 393 oocyst-positive pre-weaned calves of up to 60 days of age sampled from the province of Buenos Aires (n=99), Cordoba (n=281), and Santa Fe (n=13), Argentina, were determined to be exclusively infected by the species *C. parvum* (Tomazic et al., 2013; Del Coco et al., 2014; Lombardelli et al., 2019). Furthermore, it has been reported that *C. parvum* constitutes the predominant species in pre-weaned calves in most regions of the world, with the exception of China and Sweden where *C. bovis* prevails in this age group (Silverlås et al., 2010; Santin et al. 2013; Wang et al., 2017). Thus, it is not surprising that in the present study exclusively *C. parvum* was identified in the 35.1% oocyst-positive samples analyzed by PCR-RFLP. Considering the abovementioned results obtained in the province of Buenos Aires, Cordoba, and Santa Fe, and in most other regions (Santin et al., 2004, 2008; Brook et al., 2008), it is possible to hypothesize that all oocyst positive calves younger than 60 days (all but one of the analyzed animals, see Supplementary Table 1) were infected with *C. parvum*. Notwithstanding, the sporadic presence of other nonpathogenic *Cryptosporidium* spp. such as *C. bovis* and *C. ryanae* cannot be entirely excluded. *C. bovis* and *C. ryanae* are predominantly found in post-weaned calves, whereas *C. bovis* is usually reported in pre-weaned calves in very low frequencies, yet both these species have so far not been detected in Argentina (Santin et al., 2013).

Cryptosporidium spp. was found to be the most frequent enteropathogen in both age groups infecting 29.8% aged 1 to 20 days and 5.6% of calves aged 21 to 90 days. These results are consistent with previous reports on the prevalence of this parasite in dairy calves of the Argentine provinces of Buenos Aires (16% in 1 to 70-day-old calves, Garro et al., 2016), Cordoba (25.5% in 1 to 60-day-old calves, Lombardelli et al., 2019) and Santa Fe (19.8% in 1 to 119-day-old calves and 24% in 1 to 60-day-old calves, Aguirre et al., 2014; Modini et al., 2011). Our results combined with previous findings, support the view that *Cryptosporidium* spp. is ubiquitously present in calves of all major dairy regions of Argentina.

In the present study, every calf was sampled only once as is common for cross-sectional analyses, and allows direct comparison of our data with those of corresponding other reports. However, it has to be taken into account that, under artificial rearing conditions, all calves become infected at some time point in the preweaned phase (Zambriski et al., 2013). Thus, single sampling probably underestimates the true prevalence of *Cryptosporidium* spp. and, based on the same reasoning, this also applies to the true prevalence of diarrhea.

The frequency of *Cryptosporidium* shedding in the group of younger calves is, with 29.8 %, in a similar range as that reported from The Netherlands (27.8 % in 1 to 21-day-old calves) (Bartels et al. 2010), but considerably higher than that reported from New Zealand (15.8 % in 9 to 20-day-old calves) (Al Mawly et al., 2015a). Apart from climatic factors, this discrepancy may be due to differences in hygiene and/or calf handling during the artificial rearing stage. Importantly, zoonotic subtypes of *C. parvum* presenting a potential health risk for operators that are in direct contact with dairy calves have been reported (Tomazic et al., 2013; Del Coco et al., 2014; Lombardelli et al., 2019).

Of studied enteropathogens, RVA was the second most frequent in both age groups, found in 12.4% of calves aged 1 to 20 days and 4.0% of calves aged 21 to 90 days. The frequency of RVA in the study region is considerably lower than those previously reported (30%, 40%, and 62.5%) in other regions of Argentina (Costantini et al. 2002, Garaicoechea et al. 2006, Badaracco et al. 2012). However, in contrast to the present report, the mentioned studies correspond to diarrhea outbreaks and/or sampling of only diarrheic calves.

Compared to RVA, the proportion of calves infected with BCoV was lower in both age grups (6.4% *vs*. 12.4 % and 1.8% *vs*. 4%, for the 1 to 20 and 21 to 90-day-old groups, respectively). Surprisingly, these results are considerably higher than the frequency of 1.7% previously reported in diarrheic dairy calves in a longitudinal study over 16 years carried out in Argentina (Bok et al., 2015). Furthermore, it is worthy to note that the highest frequency of infected calves was observed for *Cryptosporidium* spp. and RVA at the second week of age, whereas for BCoV at the first week of age, this agrees with previously reported frequency distributions of enteropathogens (Bartels et al., 2010).

In this study ETEC was absent in the young calves and occurred only sporadically in the older ones (0.9 %). In the studied farms, a large proportion (25.3%) of the younger calves and 6.5% of the older ones received treatment with parenteral antibiotics. This could explain the absence and marginal frequency of ETEC in these groups, and the complete absence of *Salmonella* spp. in both (Grønvold et al., 2011). This notion is supported by a recent report of a high prevalence of 31 % of ETEC in calves of the province of Cordoba where no antibiotic treatment was carried out (Picco et al., 2015). Furthermore, a prevalence of 5.5 % of *Salmonella* spp. was reported in a study of healthy and diarrheic calves from the western region of Buenos Aires province, and treatment of diarrhea with antibiotics was likewise not reported in this study (Bilbao et al., 2019).

Importantly, in the younger age group, the observed percentage of 3.7 % of coinfection of *Cryptosporidium* spp. and RVA corresponds with that expected under conditional probability, suggesting that infection of these pathogens is stochastically independent. The same applies to the observed concurrent shedding of RVA and BCoV, determined at 0.4% and found to be similar to a reported coinfection of 0.6% by Bok et. al (2015). Although coinfection is highly significantly associated with diarrhea (bivariate analysis: Table 2), its importance in the etiology of diarrhea is negligible, since it occurs with a very low frequency under conditional probability.

A significant increase in the frequency of diarrhea was observed in the 1 to 20-day-old as compared to the 21 to 90-day-old group (28.8% *vs*.11.7%), corroborating previous reports that calf age plays a critical role in susceptibility to diarrhea (de la Fuente et al., 1999; Millemann, 2009; Bartels et al., 2010; Delafosse et al., 2015). It has been put forward that the maturation of the intestinal tract and immune system might reduce the susceptibility to infection of older calves (Kertz et al., 2017). The estimate of 28.8%

diarrheic calves in the younger group corresponds well with previously reported prevalence values of diarrhea in 22%, 27% and 36% of calves in three independent studies, two of which were carried out in the province of Cordoba and one in Buenos Aires (Bellinzoni et al., 1990; Picco et al., 2015; Lombardelli et al., 2019). Interestingly, the frequency of diarrhea in younger calves observed in the present study (28.8%) was considerably higher than the frequencies reported for diarrheic calves of similar age strata in The Netherlands (19.1 %, Bartels et al., 2010) and New Zealand (9.5 %, Al Mawly et al., 2015b). This is particularly noteworthy since the vaccination against neonatal calf diarrhea of pregnant cows applied in all farms of our study was expected to significantly reduce the incidence of diarrhea, as reported by others (Al Mawly et al., 2015a). In this context, a careful consideration of findings and observations suggests that the most important etiological factor that promotes diarrhea in dairy calves in Argentina and The Netherlands is *Cryptosporidium* spp., which is considerably more prevalent in these countries than in New Zealand.

In the present study, *Cryptosporidium* spp. shedding was found to be associated with diarrhea with very high odds (OR=5.7) in the age stratum of 1 to 20 days. Thus, this enteropathogen was the most strongly associated with diarrhea, confirming the importance of this parasite as the principal cause of calf scour as reported by Fayer et al. (1998). Higher and comparable odds for diarrhea due to *Cryptosporidium* spp. shedding were reported by Bartels et al. (2010) (*Cryptosporidium* spp.: OR=7.3) and

Trotz-Williams et al. (2007) (*Cryptosporidium* spp.: OR=5.3), whereas considerably lower odds were reported by Al Mawly et al. (2015a) (*Cryptosporidium* spp.: OR=2.6). In addition, RVA shedding was found to be associated with diarrhea in the age stratum of 1 to 20 days, though with an OR half as high as that estimated for *Cryptosporidium* spp. (OR = 2.5 *vs*. 5.7). Similar OR values were observed for RVA in studies from New Zealand (OR = 2.7) (Al Mawly et al., 2015a) and The Netherlands (OR = 2.5) (Bartels et al. 2010), reinforcing that RVA represents an additional etiological cause for the occurrence of diarrhea besides *Cryptosporidium* spp.

No association between BCoV shedding and diarrhea could be observed in the present study, which is in accordance with a number of other reports (Björkman et al. 2003; Bartels et al., 2010; Al Mawly et al., 2015a). Bartels et al. (2010) suggest that BCoV infections are opportunistic and occur when calves had previous episodes of diarrhea and had been treated against bacterial infections. Noteworthy, reports of a significant association of BCoV shedding with calf diarrhea could recently be demonstrated in an optimized case-control study design by Gomez et al., (2017).

In summary, the most frequent enteropathogen in both age groups was *Cryptosporidium* spp., followed by RVA and both pathogens increased significantly the odds of diarrhea in younger calves. Our findings allowed an integrated insight into enteropathogen epidemiology and identified their associations with diarrhea in the studied large-scale farms. This will facilitate the establishment of control measures targeting the pathogens that cause calf diarrhea under the epidemiological situation studied.

5. Conclusions

The present study determined the frequency of shedding of *Cryptosporidium* spp. and other enteropathogens and their association with diarrhea in dairy calves of Buenos Aires province, Argentina. In 1 to 20-day-old calves, *Cryptosporidium* spp. showed the highest frequency of 29.8% with high odds of 5.7 for diarrhea, followed by RVA, which exhibited a three-times lower frequency of 12.4% and about half the odds of 2.5. In contrast, BCoV in calf stool was considerably less frequent (6%) in young calves and not found to be associated with diarrhea. ETEC and *Salmonella* spp. were absent in the younger age group possibly due to the extensive use of antibiotics in the studied farms. Our study is in accordance with previous reports, which demonstrated that the frequency of enteropathogens and diarrhea is significantly lower in 21 to 90- day-old calves. Furthermore, our results suggest that, of the tested enteropathogens, the presence of *Cryptosporidium* spp. in particular may explain the relatively high prevalence of diarrhea in farms of the study region. Therefore, the implementation of additional control measures targeted primarily against *Cryptosporidium* followed by RVA should be most successful in decreasing the occurrence and impact of diarrhea in calves of dairy farms of the province of Buenos Aires, Argentina.

Funding: The study was funded by the National Animal Health Project PNSA 1115053 of the National Institute of Agricultural Technology, Argentina, the project PICT-2018-03314 of the National Agency of the Promotion of Science and Technology, Argentina, and by the project PID8-2015 of the Fundación de la Universidad de Morón.

Acknowledgments: The authors wish to thank all the participating farmers and laboratory personnel. The invaluable collaboration of veterinarians Diego Chiaravalle, Florencia Zabalaga, Hernan Finnegan, Ignacio Bustos, Juan Lagioia, Marcelo Ferrari, Maren Ebinger, Martin Carboni, Mauricio Zeleme, Natalia Weber, Nestor Formio, Nestor Osacar, Tomas Pernía, Guillermina Osacar, Juan Auzmendi and Walter Juckson is gratefully acknowledged as well as the methodological review carried out by Maria Eugenia Utges.

Authors' contributions: Conceptualization, Carlos Garro, Gabriel Morici and Leonhard Schnittger; Data curation, Carlos Garro; Formal analysis, Carlos Garro; Funding acquisition, Viviana Parreño and Leonhard Schnittger; Investigation, Carlos Garro, Gabriel Morici, Mariela Tomazic, Daniel Vilte, Micaela Encinas, Celina Vega, Marina Bok and Viviana Parreño; Methodology, Carlos Garro and Leonhard Schnittger; Project administration, Leonhard Schnittger; Resources, Viviana Parreño; Writing – original draft, Carlos Garro; Writing – review & editing, Leonhard Schnittger.

Conflict of Interest: The authors declare no conflict of interest.

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Table 1: Proportion of healthy and diarrheic calves aged 1 to 20 days and 21 to 90 days showing enteropathogen shedding

Enteropathogens[a]	Number of positive farms (%) (*n* = 42)	Number of positive calves (%)			
		1 to 20 days old		21 to 90 days old	
		with diarrhea (*n* = 139)	without diarrhea (*n* = 343)	with diarrhea (*n* = 50)	without diarrhea (*n* = 376)
Cryptosporidium spp.	31 (73.8)	69 (49.6)	75 (21.8)	5 (10.0)	19 (5)
Rotavirus	26 (61.9)	24 (17.2)	36 (10.4)	3 (6.0)	14 (3.7)
Coronavirus	14 (33.3)	10 (7.1)	21 (6.1)	1 (2.0)	7 (1.8)
Enterotoxigenic *E. coli*	4 (9.5)	0 (0)	0 (0)	0 (0)	4 (1.0)
Salmonella spp.	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Crytosporidium spp. + Rotavirus	14 (33)	13 (9.3)	5 (1.4)	0 (0)	0 (0)
Crytosporidium spp. + Coronavirus	13 (30.9)	3 (2.9)	10 (2.9)	0 (0)	0 (0)
Crytosporidium spp. + Enterotoxigenic *E. coli*	1 (2.3)	0 (0)	0 (0)	0 (0)	1 (0.2)
Coronavirus + Rotavirus	2 (4.7)	1 (0.7)	1 (0.7)	0 (0)	0 (0)

Crytosporidium spp. + Coronavirus + Rotavirus	1 (2.3)	1 (0.7)	0 (0)	0 (0)	0 (0)

[a]only pathogen combinations of which at least one case was detected are shown

Table 2: Variables associated with calf diarrhea ($p<0.2$) in bivariate screening analysis with farm identifier modelled as random effect

Variable	Categories	1 to 20-day-old calves			21 to 90-day-old calves		
Calf-level variables		OR	CI 95%	p-value	OR	CI 95%	p-value
Cryptosporidium spp.	yes/no	5.6	3.2 - 9.5	**<0.001****	2.1	0.6 - 7.5	0.232
Rotavirus	yes/no	2.3	1.2 - 4.4	**0.011***	1.4	0.3 - 6.3	0.610
Coronavirus	yes/no	1.7	0.6 - 4.8	0.260	1.6	0.1 - 17.5	0.684
Enterotoxigenic *E. coli*	yes/no		Not tested			Not tested	
Cryptosporidium spp. + Rotavirus	yes/no	9.2	2.8-29	**0.001****			
any other combination of agents observed	yes/no	1.4	0-6.3	0.653		Not tested	

significance levels: *significant and **highly significant

Table 3*:* Variables independently associated with diarrhea (p<0.05) in the final logistic regression models with random farm effect in 1 to 20-day-old calves

Variable	Coef.	OR	CI 95 %	p-value
Cryptosporidium spp.	1.7	5.7	3.3 - 9.9	**<0.001****
Rotavirus	0.9	2.5	1.2 - 5.1	**0.01***

Significance level: *significant and **highly significant

Conflict of Interest: The authors declare no conflict of interest.

Our work meets all the ethical requirements suggested https://www.elsevier.com/authors/journal-authors/policies-and-ethics) by the publisher.

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Highlights

- *Cryptosporidium* spp. was the most frequent enteropathogen in both age groups studied
- *Cryptosporidium* spp. and RVA increased significantly the odds of diarrhea in calves up to 20 days of age
- *Cryptosporidium* spp., RVA and BCoV were more frequent in younger than in calves older than 20 days