Causes of neonatal calf diarrhea and mortality in pasture-based dairy herds in Uruguay: a farm-matched case-control study

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Abstract

Neonatal calf diarrhea (NCD) and mortality cause significant losses to the dairy industry. The preweaning dairy calf mortality risk in Uruguay is high (15.2%); however, causes for these losses are largely unknown. This study aimed to assess whether various pathogens were associated with NCD and death in Uruguayan dairy calves and whether these infections, diarrhea, or deaths were associated with the failure of transfer of passive immunity (FTPI). Contemporary diarrheic ($n = 264$,) and non-diarrheic ($n = 271$) 1- to 30-day-old calves from 27 farms were sampled. Feces were analyzed by antigen-capture ELISA for *Cryptosporidium* spp., rotavirus, bovine coronavirus, and *Escherichia coli* F5+, RT-PCR for bovine astrovirus (BoAstV), and bacterial cultures for *Salmonella enterica*. Blood/serum was analyzed by RT-PCR or antigen-capture ELISA for bovine viral diarrhea virus (BVDV). Serum of ≤ 8-day-old calves ($n = 95$) was assessed by refractometry to determine the concention of serum total proteins (STP) as an indicator of FTPI. Whether the sampled calves died before weaning was recorded. At least one pathogen was detected in 65.4% of the calves, and this percentage was significantly higher in diarrheic (83.7%) versus non-diarrheic (47.6%) calves. Unlike the other pathogens, *Cryptosporidium* spp. and rotavirus were associated with NCD. Diarrheic calves, calves infected with any of the pathogens, and calves infected with rotavirus had significantly lower concentrations of STP. Diarrheic calves had higher chances of dying before weaning than non-diarrheic calves. Diarrheic calves infected with *S. enterica* were at increased risk of mortality. Controlling NCD, salmonellosis, cryptosporidiosis, and rotavirus infections, and improving colostrum management practices would help to reduce calf morbi-mortality in dairy farms in Uruguay.

Keywords *Cryptosporidium* spp. · Dairy calves · Diarrhea · Failure of transfer of passive immunity · Infectious diseases · Mortality · Rotavirus · *Salmonella enterica*

Introduction

Dairy farming in Uruguay is socio-culturally and economically important, as Uruguay is one of the top per capita consumers of dairy products in Latin America [1], and approximately 70% of the milk produced in the country is exported [2]. Uruguayan dairy farming systems are largely pasture-based, with 75% of the diet of the milking herd being

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farm-grown forage [3]. From 1985 to 2016, milk production grew linearly at a rate of 3.2% per year, with an attendant reduction in the number of farmers and the area allocated to dairy farming. This growth was due to increased stocking rates, individual milk production and the milking cow:dry cow ratio [2], indicating a production intensification process. However, the national dairy stock did not grow significantly over the past decade [2], which has partly been attributed to a relatively high (15.2%) nationwide annual dairy calf mortality risk from birth to weaning [4]. Despite this, little information is available in the scientific literature on the causes of dairy calf diseases and death in this country.

Neonatal calf diarrhea (NCD) is the leading cause of dairy calf morbidity and mortality within the first month of life [4, 5]. Affected calves suffer from dehydration, electrolyte imbalances, and metabolic acidosis, which, if left untreated, can lead to death [6]. Long-term effects of NCD in dairy heifers include reduced weight gain and development, increased time to first calving and reduced milk production in the first lactation, which result in significant economic losses to the livestock sector [7, 8]. As the leading cause of dairy calf morbidity and mortality, NCD also raises serious concerns on newborn calf welfare [9] and the excesive use of antibiotics with potential increase of antibiotic resistance [10, 11].

NCD is a complex and multifactorial syndrome, it can be caused by several infectious and parasitic agents, including viruses (e.g., rotavirus, bovine coronavirus -BCoV-, and bovine viral diarrhea virus -BVDV-), bacteria (e.g., enterotoxigenic and enteropathogenic/enterohemorrhagic *Escherichia coli* and *Salmonella enterica*), and protozoa (e.g., *Cryptosporidium* spp. and *Eimeria* spp.) [12–15], some of which are zoonotic. Although under field conditions BVDV rarely causes diarrhea in neonatal calves that receive colostral antibodies, it can be associated with perinatal and neonatal mortality due to congenital infections [16, 17]. Other agents that infect neonatal calves, such as bovine astrovirus (BoAstV), have been suspected to play a causative role in NCD, although attempts to experimentally reproduce diarrhea in gnotobiotic calves have been unsuccessful [18], and information available from field studies is limited [15]. Studying causality in spontaneous outbreaks of diseases with multifactorial etiologies is challenging and requires extensive laboratory testing for various pathogens. Agents that cause NCD can be found both in diarrheic and non-diarrheic calves, and the same or different calves in an outbreak can be coinfected by two or more causative agents [19–21], making interpretation of individual test results difficult in clinical contexts.

Additionally, NCD is frequently associated with nutritional and/or immunological factors, such as failure in the transfer of passive immunity (FTPI) [8, 22], and environmental and management factors that either favor the transmission of the causative agents [20] or increase the susceptibility of the calves. Transfer of passive immunity is arguably the single most

important non-infectious factor determining neonatal calf health and survival [23]. Calves with FTPI are at increased risk of disease and mortality [22, 24], and a large proportion of calf deaths up to 3 weeks of life can be attributed to FTPI [5, 25].

NCD outbreak investigations should be comprehensive and consider not only the infectious and parasitic etiological agents in affected and unaffected calves, but also epidemiological aspects and herd management practices that can vary greatly between farms [26]. In this context, farm-matched case-control studies represent adequate designs for the evaluation of possible associations between single or mixed infection and clinical outcomes, to better understand the causal role of the different agents while minimizing confounders. NCD has been broadly studied through cross-sectional [19, 20, 27, 28] and, to a lesser extent, case-control designs [21, 29, 30]; although these case-control studies were not matched considering the farms of origin of the calves, somewhat limiting the conclusions that can be drawn from them.

Because causes of NCD and mortality in Uruguayan dairies are largely unknown, in this study, we aimed at assessing the association of several known (*Cryptosporidium* spp., rotavirus, BCoV, *E. coli* F5+, *S. enterica*), putative (BoAstV) and occasional (BVDV) pathogens for calves with diarrhea and/or death, and whether these infections, diarrhea, or death were associated with FTPI through a farm-matched case-control study.

Materials and methods

Study design

A case-control study was conducted in 27 pasture-based, commercial dairy farms in six Uruguayan departments (San José, Río Negro, Colonia, Flores, Florida and Soriano) between January and November 2016. Farms were sampled by convenience because they were experiencing spontaneous outbreaks of NCD. In all farms, Holstein was either the only or the predominant breed of cattle; two farms had some Holstein-Jersey crosses. Contemporary calves with diarrhea (cases) and a similar number of non-diarrheic calves (controls) were sampled in each farm. Only calves aged 1–30 days were included; control calves were within an age range of 0–13 days of their respective cases. On average, 19.8 calves per farm (range: 13–29) were sampled, totaling 535 calves. Overall, 49.3% (*n* = 264) of the calves included in the study were experiencing diarrhea (cases) at the time of sampling, and the remainder 50.7% (*n* = 271) were not experiencing diarrhea and did not have a history of diarrhea (controls). The overall case:control ratio was 1:1.03. The sample size was calculated using an online epidemiological calculator [31], considering a power of 80% to detect an association between diarrhea and a

given infectious/parasitic agent, a percentage of exposed controls of 5%, and an Odds Ratio (OR) of 2.5 with a 95% confidence level.

Animal specimens

Individual fecal, whole blood, and serum samples were obtained from each calf by a veterinarian, following procedures approved by INIA´s animal ethics committee for the use of animals in experimentation (protocol #20199). Fecal samples were collected from the rectum using individual sterile fecal cups and gloves. A fecal score was assigned to each sample as previously described [32], based on which calves were classified as either non-diarrheic (controls; fecal score ≤ 1) or diarrheic (cases; fecal score ≥ 2). Additionally, the fecal samples were assessed macroscopically for the presence of fibrin and/or mucus. Blood samples were drawn by jugular venipuncture using individual sterile needles and syringes and collected in red top tubes for serum and heparinized tubes (BD Vacutainer, Franklin, NJ) for whole blood.

Data collection

At the end of the calf-rearing period in each of the 27 farms, a questionnaire was conducted to the farmers to assess whether the individual calves sampled for this study had died within the rearing period (before weaning), until 60 days of age (follow-up time), and the age in days at death, when available (Supplementary Material 1).

Pathogen detection in feces

Enzyme-linked immunosorbent assay (ELISA) for coproantigen detection

A commercial monoclonal antibody-based antigen-capture ELISA kit (Pathasure Enteritis 4, Biovet Inc., St-Hyacinthe, Canada) was used to detect *Cryptosporidium* spp., rotavirus, BCoV and *E. coli* F5+ (K99+) antigens in fresh feces from all 535 calves [33], 24–72 h after sample collection, following the manufacturer´s recommendations.

Salmonella enterica culture and serotyping

All 535 fecal samples were cultured aerobically in tetrathionate broth (Oxoid, code CM0671) for 24–48 h at 37°C (selective enrichment) after which 100 µl of broth were plated onto xylose-lysine-deoxycholate (XLD) agar (Oxoid, code CM0469). Suspect colonies were selected, and routine biochemical tests were performed for identification of *S. enterica*, as previously described [34]. *Salmonella enterica* serotyping was performed following the Kauffman-White-Le Minor classification scheme [35] at the bacteriology service of

the "Instituto de Higiene, Facultad de Medicina, Universidad de la República," in Montevideo, Uruguay.

RT-PCR for bovine astrovirus

A total of 396 fecal samples were diluted 1:10 (v:v) in phosphate-buffered saline solution and centrifuged at 3000*g* at 4°C for 20 min. Supernatants were collected and stored in a freezer at −80°C. Viral RNA was extracted using QIAamp cador Pathogen Mini Kit (Qiagen) with an elution volume of 50 µL. Reverse transcription (RT) was carried out with RevertAid Reverse Transcriptase (ThermoFisher, Scientific) and random hexamer primers (Qiagen) to obtain cDNA that was stored at -20°C. BoAstV PCR was performed using MangoMix (Bioline) and primers BoAstV-F and BoAstV-R that amplify a 432-nucleotide fragment of the polymerase gene of BoAstV, as described elsewhere [36]. PCR products were visualized in 2% agarose gels. The results were expressed as positive or negative.

Bovine viral diarrhea virus detection in blood or serum

Frozen samples of serum (*n* = 418) or whole blood (*n* = 62) were processed for BVDV detection, either by a commercial antigen-capture ELISA (BVDV Ag/Serum Plus Test, IDEXX, Switzerland) (136 serum and 62 whole blood samples) or by RT-PCR (282 serum samples). The RT-PCR was performed as previously described [37].

Assessment of transfer of passive immunity in neonatal calves

Serum samples from all calves that were ≤ 8 days of age at the time of sampling (*n* = 95) were analyzed for serum total solids using an optic refractometer (ATAGO PAL-1, Tokyo, Japan), as an indicator of the concentration of serum total proteins (STP) in g/dl. A cutoff value of < 5.6 g/dl was considered to determine FTPI, as previously described [38]. Additionally, the results were categorized to reflect calves with poor (< 5.1 g/dl), fair (5.1–5.7 g/dl), good (5.8–6.1 g/dl), or excellent (> 6.2 g/dl) transfer of passive immunity as suggested by Godden et al. [23].

Data and statistical analyses

Data of each sampled calf, including the age at sampling, farm of origin, occurrence of diarrhea, presence or absence of fibrin and/or mucus in feces, all laboratory test results (pathogen detection, STP concentration), and the information from the questionnaire, including whether calves had died or survived during the preweaning period (follow-up time) was collected. Data was entered into a Microsoft Excel 2013 spreadsheet to

create a digital database (Supplementary Material 1) that was used as a template for statistical analyses. A brief description of the statistical analyses are presented in the following paragraphs; a more detailed description is available in Supplementary Material 2.

Descriptive statistics of the raw data including proportions, means, and standard deviations (SD, for data with normal distribution), median and interquartile range (IQR, for data not normally distributed) were calculated. Differences between the proportions of diarrheic calves by age in weeks, the proportions of diarrheic and non-diarrheic calves that tested positive to at least one of the pathogens and to ≥ 2 pathogens, and the proportions of diarrheic and non-diarrheic calves that died before weaning were assessed by chi-square. Differences in the STP concentrations (g/dl) in diarrheic versus non-diarrheic calves, as well as calves that tested positive or negative for (a) given pathogen(s) were evaluated by analyses of variance using the procedure PROC MIXED (SAS University Edition, SAS Institute Inc. Cary, NC, USA), controlling for herd as a random effect. Results were expressed in least squares means (LMS) and standard errors of the means (SEM).

A logistic regression model was fit to assess the effect of the pathogens on diarrhea. The model accounted for the structure of the sample design in which diarrheic calves (cases) were matched with control calves within a farm [39]. The variable "age" grouped in weeks (1, 2, 3, and > 3) was included in the model to adjust the OR. Interaction terms between the pathogens were tested to assess the effects of co-infections. The model was made using the procedure PROC LOGISTIC including the farm identification in the STRATA statement; the model fit was checked by the Akaike Information Criterion (AIC) and r squared [22, 40, 41].

A univariate generalized estimating equation (GEE) repeated-measures logistic model was made to assess the effect of the concentration of STP on diarrhea, rotavirus, BCoV, and *E. coli* F5+ detection adjusted by the age of the calves in days. This hypothesis was tested because vaccination against these pathogens is a common practice in Uruguayan dairy farms. For this model, values of STP concentration < 5.6 g/dl were classified as low and compatible with FTPI, as previously described [38].

A multivariate GEE repeated-measures logistic model was employed to assess the association between the presence of fibrin or mucus in the feces and the pathogens adjusted by age of the calves in weeks.

Finally, we tested the risk of death among diarrheic calves infected with *S. enterica*, rotavirus and *Cryptosporidium* spp. adjusted by age in days using the same multivariate GEE model structure but including the Poisson instead of the binomial distribution to estimate the risk of death [42]. BCoV, BoAstV, BVDV, and *E. coli* F5+ were not included because there were either only one (BCoV, BVDV and *E. coli* F5+) or too few (BoAstV) deceased diarrheic calves infected with

these pathogens. For this, data from all the diarrheic calves for which the questionnaire was available (118 calves) was used; these calves were followed-up until the end of the pre-weaning period.

A significance level (alpha) of 5% ($p < 0.05$) was considered for all the statistical analyses.

Results

At the time of sampling, the calves had a median of 11 days of life (IQR = 9), with a range of 1 to 30 days [non-diarrheic calves = 12 (IQR = 14), diarrheic calves = 11 (IQR = 5)]. The proportion of diarrheic calves was 39.9% (61/153) in the first week of life, 71.8% (158/220) in the second, 28.9% (33/114) in the third and 25.0% (12/48) after the third week of life. The proportion of diarrheic calves was significantly higher in the second week of life ($p < 0.001$). Fibrin or mucus were observed in the feces of 8.1% (21/259) and 47.1% (122/259) of diarrheic calves and 2.6% (7/266) and 32.3% (86/266) of non-diarrheic calves, respectively.

The questionnaire was completed for 241 calves (118 cases and 123 controls), 19.9% (48/241) of which had died before weaning at 5–37 days of age (median age at death: 16, IQR = 13). The remainder 80.1% (193/241) of the calves survived after weaning (> 60 days of age). Thirty-one (64.6%) of the calves that died before weaning were diarrheic, and the remainder 35.4% (17/48) were non-diarrheic at the time of sampling. Of the 193 calves that survived after weaning, 45.0% (87/193) were diarrheic, and the remainder 55.0% (106/193) were non-diarrheic at the time of sampling. The proportion of calves that died before weaning was significantly higher among diarrheic than non-diarrheic calves ($p < 0.02$). Individual results for each calf in each farm are shown in Supplementary Material 1.

Detection of pathogens in feces and BVDV in serum/blood

At least one of the pathogens was detected in 65.4% (350/535) of the calves, this percentage was significantly higher in diarrheic (83.7%, 221/264) versus non-diarrheic (47.6%, 129/271) calves ($p < 0.001$). In 34.6% (185/535) of the calves no agents were detected, being 76.8% (142/185) of them non-diarrheic and the remaining 23.2% (43/185) diarrheic ones. Individual pathogens and coinfections were identified in 43.2% (231/535) and 22.2% (119/535) of the calves, respectively. The frequencies of detection of each pathogen individually and in coinfections are shown in Table 1 and Supplementary Material 1. *Cryptosporidium* spp., BoAstV and rotavirus were the most frequently detected agents, both at the animal and farm levels (Table 1). *Cryptosporidium* spp. and rotavirus were detected with a significantly higher

Table 1 Overall frequency of detection of pathogens in 535 diarrheic and non-diarrheic dairy calves from 27 farms in Uruguay

Tests (sample type and total No. analyzed)	No. of samples analyzed from diarrheic and non-diarrheic calves	Total no. (%) of positive calves	Total no. (%) of diarrheic positive calves	Total no. (%) of non-diarrheic positive calves	No. of farms with ≥ 1 positive animal (% of total No. of farms, $n = 27$)
Cryptosporidium spp. ELISA (feces, 535)	264 and 271	256 (47.8%)	183 (69.3%)	73 (26.9%)	27 (100.0%)
BoAstV RT-PCR (feces, 396)	186 and 210	86 (21.7%)	39 (21.0%)	47 (22.4%)	21 (77.8%)
Rotavirus ELISA (feces, 535)	264 and 271	94 (17.6%)	64 (24.2%)	30 (11.1%)	24 (88.9%)
Salmonella enterica isolation (feces, 535)	264 and 271	21 (3.9%)	14 (5.3%)	7 (2.6%)	8 (29.6%)
Escherichia coli F5+ ELISA (feces, 535)	264 and 271	11 (2.1%)	6 (2.3%)	5 (1.8%)	5 (18.5%)
BCoV ELISA (feces, 535)	264 and 271	8 (1.5%)	5 (1.9%)	3 (1.1%)	5 (18.5%)
BVDV antigen ELISA or RT-PCR (serum/blood, 480)	231 and 249	6 (1.3%)	4 (1.7%)	2 (0.8%)	4 (14.8%)

ELISA enzyme-linked immunosorbent assay, *BoAstV* bovine astrovirus, *RT-PCR* reverse transcriptase polymerase chain reaction, *BCoV* bovine coronavirus, *BVDV* bovine viral diarrhea virus

frequency in diarrheic calves, in contrast to BoAstV, which as a single infection had a significantly higher frequency in non-diarrheic calves. The proportion of diarrheic calves infected with one pathogen was 59.3% (137/231). In the 119 calves with coinfections (84 cases and 35 controls), two (89.1%; 106/119) or three (10.9%; 13/119) pathogens were identified. A proportion of 69.8% (74/106) of the calves coinfected with two pathogens were diarrheic, while the remainder 30.2% (32/106) were non-diarrheic. Of the 13 calves coinfected with three pathogens, 76.9% (10/13) were diarrheic. The proportion of diarrheic calves coinfected with ≥ 2 pathogens was 70.6% (84/119). All isolated *Salmonella* strains were *S. enterica* subspecies *enterica* serotype Typhimurium, except for two isolates from one farm that were serotype Anatum.

Association between pathogens, diarrhea, and preweaning death

In the final multivariate conditional logistic regression model individual infections with *Cryptosporidium* spp. (OR = 1.65, 95% CI = 1.25–2.17, $p = 0.0004$), rotavirus (OR = 1.79, 95% CI = 1.36–2.35, $p < 0.0001$), and their interaction ($p = 0.0274$) were associated with diarrhea. No association was observed between diarrhea and infection with BCoV, *E. coli* F5+ or *S. enterica*. There was an interaction effect between *Cryptosporidium* spp. and rotavirus, which means that the effects of one of this pathogens on diarrhea differered according with the infection status by the other and viceversa. The odds of diarrhea were higher in calves infected with *Cryptosporidium* spp. that were negative for rotavirus (OR = 5.77, 95% CI = 3.47–9.61), and in calves infected with rotavirus that were negative for *Cryptosporidium* spp. (OR = 4.93,

95% CI = 2.31–10.54), than in calves that were positive for both agents. The model with interaction terms had the best fit compared with the full model and with the model without interaction according to the AIC (507.6) and r squared values (0.35). Calves in the second week of life had significantly higher odds of being diarrheic (OR = 2.16, 95% CI = 1.25–3.69, $p < 0.0001$), while the odds decreased in calves in the third week of life (OR = 0.6, 95% CI = 0.33–1.11, $p < 0.03$). There were no associations between diarrhea and infection with BVDV (OR = 0.55, 95% CI = 0.08–3.70, $p = 0.54$) or BoAstV (OR = 0.85, 95% CI = 0.48–1.50, $p = 0.58$).

Calves without diarrhea at the time of sampling had lower chances of dying before weaning (OR = 0.40, 95% CI = 0.19–0.84, $p < 0.02$) than diarrheic calves.

Pathogens and the presence of fibrin or mucus in feces

Individual infections with *S. enterica* and rotavirus were significantly associated with the presence of fibrin in the feces in the multivariate repeated-measures logistic model. The odds of *S. enterica*-positive calves presenting fibrin in the feces was 5.8 times greater than for *S. enterica*-negative calves (OR = 6.8, 95% CI: 2.4–18.9, $p < 0.001$), while the odds of rotavirus-positive calves presenting fibrin in the feces was 1.2 times greater than for rotavirus-negative calves (OR = 2.2, 95% CI: 1.1–4.4, $p = 0.03$). None of the evaluated pathogens were associated with the presence of mucus in the feces.

Transfer of passive immunity, diarrhea, pathogens, and preweaning death

The STP concentration was assessed in 95 ≤ 8-day-old calves with (*n* = 40) and without (*n* = 55) diarrhea, 45 of them (47.4%) had FTPI based on the cutoff proposed by [38] (STP < 5.6 g/dl). According to the four-level categorization suggested by Godden et al. [23], 28.4% (27/95), 26.3% (25/95), 13.7% (13/95) and 31.6% (30/95) of the calves had poor, fair, good, or excellent transfer of passive immunity, respectively. The overall mean STP concentration was 5.64 g/dl (SD = 0.9, min = 4, max = 8.6), the LSM for the STP concentration was 5.81 g/dl (*n* = 55, SEM = 0.20) in non-diarrheic calves, and 5.36 g/dl (*n* = 40, SEM = 0.19) in diarrheic calves, these differences were statistically significant (*p* = 0.0445). Similarly, the STP concentration was significantly lower in calves infected with any of the pathogens under study (*n* = 62, LSM = 5.40 g/dl, SEM = 0.16) than in those that were negative to all tested pathogens (*n* = 33, LSM = 6.08 g/dl, SEM = 0.21) (*p* = 0.0016). Although calves with concentrations of STP ≥ 5.6 g/dl had 54.0% lower odds of presenting diarrhea than calves with STP concentrations < 5.6 g/dl (OR = 0.46, 95% CI = 0.21–1.03, *p* = 0.059), this difference was not statistically significant.

The calves that tested positive only for rotavirus had significantly lower concentrations of STP (*n* = 13, LSM = 5.20 g/dl, SEM = 0.24) than the negative ones (*n* = 82, LSM = 5.67 g/dl, SEM = 0.16) (*p* = 0.0469). Calves with STP concentrations ≥ 5.6 g/dl had 64.0% lower odds of being positive to rotavirus than calves with concentrations of STP < 5.6 g/dl (OR = 0.36, 95% CI = 0.12–1.07, *p* < 0.07), this difference was not statistically signicant. There were no associations between the STP concentration and infections by BCoV or *E. coli* F5+, or with preweaning death.

Pathogens and risk of death during the preweaning period in diarrheic calves

The risk of death before weaning was 0.9 times higher in diarrheic *S. enterica*-positive calves than in diarrheic *S. enterica*-negative ones (RR = 1.90, 95% CI = 1.13–3.16, *p* = 0.015). The other evaluated pathogens were not associated with increased risk of death of diarrheic calves before weaning.

Discussion

NCD is one of the leading causes of death in dairy calves [5]. In coherence with this, in our study, calves that manifested diarrhea early in their lives had significantly higher chances of dying before weaning than contemporary non-diarrheic ones, indicating that controlling and preventing NCD regardless of its cause would aid in reducing calf mortality in Uruguayan dairy farms. As a multifactorial syndrome associated with various infectious and parasitic agents, as well as non-infectious factors [13], determining its etiology is a complex process that usually requires laboratory testing along with clinical and epidemiological investigations. In this work, the frequency of infection with various known or putative pathogens, and their association with NCD, the presence of fibrin or mucus in feces, FTPI and death, was evaluated through a large-scale farm-matched case-control study in contemporary calves with and without diarrhea. Case-control studies are key to identify the possible associations and the role of the agents in disease, since most of the causative agents of NCD can be found in clinically healthy calves, and therefore their mere presence does not warrant disease causality. Additionally, because NCD is a multifactorial syndrome, the interaction between different potential etiologies needs to be considered in studies aiming at assessing causality. Case-control studies with large numbers of dairy calves that investigate multiple possible etiologies of NCD and conduct statistical analyses to assess interactions between multiple agents to obtain robust and reliable epidemiological information are scant in the scientific literature [21, 29, 30].

In our study, at least one of the evaluated pathogens was detected in 65.4% of the 535 calves, and 83.7% of the 264 diarrheic calves. The attributable factor in exposed calves was 0.82 (not shown), meaning that 82% of the NCD cases may be attributed to the studied agents. This also suggests that other infectious (i.e., attaching and effacing -enteropathogenic and enterohemorrhagic- *E. coli*) or non-infectious (i.e., nutritional) factors not assessed in this study, may also be contributing to NCD in a smaller proportion of cases. In our study, the proportion of diarrheic calves was higher in the second week of life, which indicates that calves are exposed to diarrheagenic agents early after birth, as observed by other authors [20, 43]. A decline in the levels of colostral neutralizing antibodies in the intestine is the main determinant for the occurrence of infectious diarrhea during the second week of life [44, 45].

The most frequently detected agents were *Cryptosporidium* spp., BoAstV and rotavirus, both as individual infections and coinfections. This is remarkable given the high frequency of detection of these agents at the farm level. The detection frequencies of rotavirus in diarrheic (24.2%) or non-diarrheic (11.1%) calves in our study were much lower than those reported by RT-qPCR by Castells et al. [46] in the same country using mostly the same sample set (72.1% and 59.9%, respectively). In our study, this frequency is probably underestimated considering that the detection limit and the sensitivity of the antigen-capture ELISA are lower than RT-qPCR, mainly in subclinical infections, and neutralizing antibodies derived either from colostrum or active immune responses may interfere with viral detection by antigen-capture ELISA [47]. Given the high frequency of these pathogens,

transmission routes within and between farms need to be further studied locally to better understand their epidemiological cycle. Rotavirus and *Cryptosporidium* spp. are highly resistant to environmental conditions; calves get infected by contact with feces from dams, which shed these pathogens subclinically contaminating the udder or calving areas. As calves are the main biological amplifyers of these enteric pathogens, transmission between calves occurs by direct contact in communal pens, or indirect contact by fecal contamination of rearing utensils [48]. Waterborne transmission seems plausible, considering that water is the main transmission route for *Cryptosporidium* spp. and has also been suggested for group A rotavirus in dairy calves in Uruguay [49], and that most dairy farms in the country administer untreated underground water or surface water to livestock. Given the geographic proximity of the dairy calf rearing areas and natural surface watersources in Uruguay, the dense network of rivers and the relatively high annual rainfalls and occasional flooding events in this country, calves pose a risk for surface water contamination with fecal pathogens, notably *Cryptosporidium* spp. [50]. Because some subtypes of *Cryptosporidium parvum* are zoonotic and cattle are reservoir of potentially zoonotic strains [51, 52], and considering that *Cryptosporidium* spp. has been identified as a cause of diarrhea in children in Uruguay [53], we further speciated and subtyped the cryptosporidia detected in calves in this study. Interestingly, of seven *C. parvum* subtypes detected in 166 calves, five subtypes detected in 143 calves from nearly all farms had been detected in humans elsewhere and have zoonotic potential [50].

Regarding coinfections and interactions between *Cryptosporidium* spp. and rotavirus, both agents were associated with diarrhea in calves that had individual infections with either pathogen, and in those that were coinfected. However, an unexpected finding was that in coinfected calves the odds of diarrhea were lower than in those infected with either pathogen. Because *Cryptosporidium* spp. and rotavirus are both intracellular pathogens that invade and affect the same target cells (superficial enterocytes) of the small intestine [54, 55], resulting in similar lesions, it can be speculated that they occupy the same cellular or subcellular niches (i.e., receptors, signaling pathways), and/or that infection with one of them somehow interferes with the ability of the other to cause further intestinal damage and diarrhea. A study in mice experimentally infected with *C. parvum* and a strain of *Enterococcus faecalis* administered as a probiotic demonstrated that when both agents were present in the same intestinal location, the bacterium interfered with *C. parvum* infection [56].

In humans, astroviruses cause acute infantile diarrhea [57]; however, the clinical relevance of enteric astroviruses in cattle is not entirely clear. A recent review on viral enteritis in calves concluded that it is currently unclear whether BoAstV is a relevant primary pathogen, a potential cause of disease with coinfections or a clinically irrelevant virus [15]. To the best of our knowledge, ours is the first work in which the possible role of BoAstV was evaluated as one of the agents of NCD and calf mortality in a farm-matched case-control study considering multiple etiologies in the study design and statistical analyses. Despite the relatively high frequency of BoAstV infection (21.7% of the calves and 77.8% of the farms), no association with diarrhea or disease was observed in our study, as suggested by Sharp et al. in Scotland [58]. Conversely, in our study, the frequency of BoAstV infection as an individual agent was significantly higher in non-diarrheic than diarrheic calves (27 of the 31 calves that were only infected with BoAstV were non-diarrheic). This not only calls into question the causative role of enteric BoAstV in NCD, but also suggests a possible beneficial infection with a protective effect on diarrhea which should be further explored. Recent molecular studies by our group indicate a high genetic diversity for BoAstV infecting dairy calves in Uruguay, including three different *Mamastrovirus* species, the most frequent of which represented an unclassified species [59]. Whether cattle harbor *Mamastrovirus* species with zoonotic potential, or whether enteric BoAstV share similarities with a neurotropic BoAstV recently identified as a cause of encephalitis in cattle in this country [60] needs further investigation.

Although the role of BCoV and BVDV as causes of diarrhea in cattle is well documented [13, 14, 61], these viruses were not associated with NCD in our study. The lack of association may have been related to the low frequency of detection, as indicated in other works carried out in various countries [19–21]. However, it should be considered that both agents can cause either enteric (BCoV and BVDV) or systemic disease (BVDV) in older cattle. We have occasionally diagnosed diseases and mortalities caused by BVDV in 3- to 4-month-old heifers [61] and BCoV in neonate calves (unpublished data) in dairy cattle in Uruguay through pathologic examinations and molecular virology, and also detected BVDV in aborted dairy fetuses [61]. In our study, 1.5% of the calves and 18.5% of the farms were positive for BCoV, and 1.3% of the calves and 14.8% of the farms were positive for BVDV, demonstrating the circulation of these viruses at an early age in dairy calves. However, Castells et al. 2019 reported a higher detection rate (7.7%) of BCoV in feces of neonate calves by RT-qPCR [62]. As with rotavirus, BCoV antigen-capture ELISA has a lower sensitivity and limit of detection than RT-qPCR, mainly in subclinical infections, and neutralizing antibodies derived from colostrum or active immune responses may interfere with viral detection by antigen-capture ELISA [47, 63]. Thus, the frequency of BCoV detection in our study is probably underestimated; in this context, the lack of association between BCoV and diarrhea could have been a consequence of the low detection frequency. Regarding BVDV, the relatively low frequency of detection at the calf level was not unexpected, as BVDV rarely causes diarrhea in neonatal calves under field conditions.

Additionally, it should be mentioned that the antigen-capture ELISA performed to detect BVDV in 136 of the 480 calves analyzed for this virus, is not suggested to detect BVDV in sera from persistently infected calves, mainly those younger than 3 months of age in which specific colostrum-derived antibody titers are moderate or high [64, 65]. However, considering that 14.8% of the farms had at least one BVDV positive calf either by antigen-capture ELISA or RT-qPCR, and that BVDV can be particularly responsible for severe economic losses [66], further investigations are needed to assess the impact of this virus to the local livestock sector.

Salmonella enterica was detected in 3.9% of the calves (14 cases and 7 controls) and 29.6% of the farms in this study, these proportions are probably underestimated considering the relative low sensitivity and high specificity of the selective culture for this agent [67]. Even though the proportion of infected animals was more than double in diarrheic versus non-diarrheic calves, the agent was not statistically associated with diarrhea. However, *S. enterica* infection was associated with the presence of fibrin in the feces of the calves. *Salmonella enterica* causes severe intestinal lesions, as well as invasive/septicemic infections leading to death [14]. The intestinal damage induced by this bacterium can be so severe to result in fibrin exudation into the intestinal lumen (fibrinous/necrotizing enteritis/enterocolitis) [68], even without or before manifestation of diarrhea. The presence of fibrin in the feces is suggestive, though not exclusive, of enteric salmonellosis, and should prompt the veterinary practitioners to establish an early medical treatment to avoid calf mortality.

The association between rotavirus infection and the presence of fibrin in feces in our study was unexpected. From a pathologic standpoint this virus causes superficial enterocyte lysis and exfoliation in the small intestine (mostly jejunum and ileum) resulting in shortening, bluntin and fusion of the intestinal villi (atrophic enteropathy) [14], which is unlikely to result in significant extravasation of fibrin from the propial blood vessels into the intestinal lumen, unless there are secondary bacterial complications leading to ulceration. We did not find multicollinearity problems or confusion bias between rotavirus and *S. enterica* infection and the presence of fibrin in feces based on the statistical test parameters, suggesting that this is not an spurious association between these two pathogens. However, other bacterial pathogens that may cause severe intestinal, colonic and/or cecal damage such as attaching and effacing -enteropathogenic and enterohemorrhagic- *E. coli* , were not assessed for in this study. A study on virulence genes of *E. coli* isolated from diarrheic and non-diarrheic dairy calves in Uruguay, using samples of the dairy calves of this study, found a poor representation of genes associated with the shiga toxin-producing *E. coli* (STEC) / enterohemorrhagic *E. coli* (EHEC) group, as well as enteropathogenic *E. coli*

(EPEC) [69]. Interestingly, a study on postmortem findings and laboratory-based diagnosis of causes of death in dairy calves in the USA found that calves with necrotizing and ulcerative intestinal lesions were more likely to be diagnosed with rotavirus infection [70].

All diarrheic *S. enterica*-positive calves that died before weaning did so within 3 days of sampling at ages that ranged between 9 and 18 days, while non-diarrheic *S. enterica*-positive calves, all of which survived after weaning at least until day 122 of age (101 days after sampling, data not shown). The diarrheic *S. enterica*-infected calves in our study had a significantly higher risk of dying during the preweaning period than the diarrheic calves not infected with this agent. Thus, salmonellosis should be considered a significant cause of calf mortality in the rearing period in dairy farms in Uruguay. Besides its impact on animal health, the role of cattle as sources of human salmonellosis should be further studied, as *S. enterica* has been recognized as a human pathogen in this country [53, 71], where the cattle population per capita is the highest in the world [72]. The predominant *S. enterica* serotype in our study was *S.* Typhimurium (19 calves in 7 farms), followed by *S.* Anatum (2 calves from the same farm). Because antibiotic resistance in animal and human pathogens is of major global concern and multi-drug-resistant *S.* Typhimurium strains have been identified in human patients in Uruguay [71], we assessed antibiotic susceptibility of all the *Salmonella* strains obtained in this study [73]. The minimum inhibitory concentration to 14 antibiotics in 9 antibiotic classes was assessed by microdilution. All 21 strains were resistant to at least one antibiotic class, and 11/21 strains were resistant to ≥ 3 antibiotic classes (predominantly tetracyclines, aminoglycosides and beta-lactams), and were thus considered multi-drug resistant strains [73]. The phenotypic and molecular bases for antibiotic resistance need to be further explored.

E. coli F5+ was found in a relatively low frequency at the individual (2.1% of the calves) and farm (18.5%) levels and was not associated with NCD. The low frequency of *E. coli* F5+ in neonatal calves had been previously documented in the region [74, 75], as well as in other parts of the world [12, 20, 21]. It should be considered that enterotoxigenic *E. coli* affects calves < 1 week of age [76], and our sampling frame included calves up to 4 weeks of age, which probably represents a bias. In our study, of the 11 calves that tested positive for *E. coli* F5+, 9 were ≤ 5 days of life and the remainder two were 12 and 15 days old. No association with diarrhea was found even when only calves within the first week of life were considered for the statistical analysis (not shown). Furthermore, it should be considered that even though the expression of the fimbrial antigen F5 and the production of heat-stable toxin (STa) are highly associated in enterotoxigenic *E. coli*, some studies have found *E. coli* F5+ strains without toxigenic potential (i.e., PCR negative

for the STa-encoding gene). This could explain why diarrhea was not observed in some of the positive calves [77–80]. In addition, it is not unexpected to have false negative results using antigen-capture ELISA for F5, as this test has a low sensitivity [12].

Determining the STP concentration in serum is an indirect way of assessing the immune status of the calves, particularly the transfer of passive immunity (colostral antibodies) in the first week of life [23, 38]. Low concentrations of STP have been associated with increased morbidity and mortality [5, 28]. In our study, almost half of the calves (47.4%) sampled for STP determination had values < 5.6 g/dl consistent with FTPI. A higher concentration of STP was observed in non-diarrheic calves, as well as in calves that were negative for rotavirus. Calves with higher STP concentrations had lower odds of manifesting diarrhea as well as being positive for this pathogen. This suggests that higher STP concentrations, indicative of successful transfer of passive immunity, may have had a protective effect against diarrhea and rotavirus infection. Vaccines available to prevent NCD usually contain rotavirus and other various viral and bacterial antigens (sometimes including BCoV and *E. coli* F5+, depending on the manufacturing laboratory) and aim at increasing specific colostral immunity in the dams, so they require adequate colostrum management practices to warrant successful transfer of immunity to protect the calves. Whether rotavirus antigens included in the vaccines available in Uruguay (all of which consist of inactivated virus) protect against the predominant viral strains needs to be addressed and deeper antigenic characterizations of local rotavirus strains should be performed. Currently, there are no commercially available vaccines for *Cryptosporidium* spp.; however, it has been postulated that calves with an adequate immune status, acquired through colostrum, are less likely to shed this agent in feces [81] and to have clinical cryptosporidiosis [82]. Interestingly, in our study, the mean STP concentration was higher in *Cryptosporidium* spp.-negative (5.71 g/dl, n = 66) than -positive calves (5.47 g/dl, n = 29) (data no shown). Although this difference was not statistically significant, these mean STP concentrations were above and below, respectively, of the cutoff value to determine FTPI [38]. This highlights the importance of applying readily available and cost-effective management practices, such as vaccination of the dams to obtain quality colostrum and its early administration to calves to prevent NCD. Although vaccination to prevent NCD is recommended in all dairy farms, it is most meaningful in those where colostrum management practices are adequate. Unfortunately, inadequate colostrum management practices are widespread among dairy farms in Uruguay [4], which may partially explain the high percentage of calves with low STP concentrations found in our study. Regarding the lack of statistical association between STP and preweaning death, it should be noted that among 31 calves with STP values < 5.6 g/dl, 14 had missing data on

preweaning death. In addition, 22 calves with STP values ≥ 5.6 g/dl had missing data on preweaning death (Supplementary Material 1). This represents 37.9% (36/95) of calves with missing data, which probably influenced the outcome of the statistical analysis.

Conclusions

We generated reliable epidemiological information to apply specific control and preventive measures to reduce NCD associated losses in Uruguay. This was achieved through a farm-matched case-control study, evaluating multiple enteropathogens, clinical signs, preweaning death, FTPI, and their associations. We conclude that NCD is an important cause for mortality of dairy calves in Uruguay, regardless of its cause. *Cryptosporidium* spp. and rotavirus cause NCD and are frequent both at the calf and farm levels. *Salmonella enterica* infection results in fibrinous stools and increases the risk of preweaning mortality in diarrheic calves. BoAstV, despite being frequent, is not a primary cause of diarrhea for dairy calves in Uruguay. Although the frequencies of BCoV, BVDV and *E. coli* F5+ are relatively low, these pathogens are probably underestimated and their role in neonatal disease should not be disregarded. The STP concentration in neonate calves has a protective effect against diarrhea and rotavirus infection, demonstrating the importance of applying adequate colostrum management practices to improve neonatal calf health and well-being and reduce diarrhea-associated mortality. Lastly, neonatal dairy calves in Uruguay are reservoirs of potentially zoonotic pathogens, notably *Salmonella* Typhimurium and *Cryptosporidium* spp. that have been identified in human patients in this country.

Author contribution RDC, MF, FRC, and FG conceived the study. RDC, MLC, and COS performed field work. RDC, MLC, COS, MC, LM, and RC conducted laboratory testing. RDC and LGC analysed data. RDC and FG wrote the first draft and final version of the manuscript. All authors read, edited, and approved the final version of the manuscript.

Innovación" (ANII), N-15156_PL_15_0_00 and PL_27 N-23398 from INIA, and INI_2017_158 from "Comisión Sectorial de Investigación Científica" (CSIC) of the "Universidad de la República" (UdelaR).

Declarations

Ethics approval Procedures involving sampling of calves were reviewed and approved by INIA´s animal ethics committee for the use of animals in experimentation (protocol #20199).

Consent to participate All authors gave their consent to participate.

Consent for publication All authors gave their consent for the publication of the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

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