Title

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Journal

Brazilian Journal of Microbiology, 54(1)

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Publication Date

2023-03-01

DOI

10.1007/s42770-022-00898-9

Peer reviewed

Brazilian Journal of Microbiology (2023) 54:475–490
https://doi.org/10.1007/s42770-022-00898-9

VETERINARY MICROBIOLOGY - RESEARCH PAPER

Epidemiological and clinicopathological findings in 15 fatal outbreaks of salmonellosis in dairy calves and virulence genes in the causative *Salmonella enterica* Typhimurium and Dublin strains

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Received: 6 December 2021 / Accepted: 20 December 2022 / Published online: 5 January 2023

Abstract

Salmonella enterica is a major food-borne pathogen that affects cattle-rearing systems worldwide. Little information is available on the epidemiology and pathology of salmonellosis and the virulence genes (VGs) carried by *Salmonella* in spontaneous outbreaks in cattle. We describe epidemiological findings in 15 fatal outbreaks of salmonellosis in Uruguayan dairy farms and the age, clinical signs, and pathology in 20 affected calves. We also describe the serotypes and frequencies of 17 VGs in the causative *Salmonella* strains and explore their associations with epidemiological, clinical, and pathological findings. *Salmonella* Typhimurium and Dublin were identified in 11/15 and 4/15 outbreaks, respectively. The most frequent reason for consultation was digestive disease (8 outbreaks caused by *S*. Typhimurium), followed by sudden death (4 outbreaks, 3 caused by *S*. Dublin). Morbidity, mortality, and lethality ranged 4.8–100%, 3.8–78.9%, and 10–100%, without significant differences between serotypes. Diarrhea, the most common clinical sign (14 cases), was associated with the Typhimurium serotype (OR = 26.95), especially in ≤ 30-day-old calves with fibrinous enteritis as the main autopsy finding. The Dublin serotype affected ≥ 50-day-old calves and was associated with fibrinosuppurative splenitis ($p = 0.01$) and tubulointerstitial nephritis (OR = 48.95). The chances of the Dublin serotype increased significantly with age. There was low variability of VG across serotypes. The *pef*A gene was associated with the Typhimurium serotype (OR = 21.95), macroscopic enteritis ($p = 0.03$), and microscopic fibrinosuppurative splenitis ($p = 0.04$). Understanding the epidemiology, pathology, and virulence of *S. enterica* at the farm level is key to delineating prevention and control strategies to mitigate its impact on animal and human health.

Keywords *Salmonella enterica* · Calves · Epidemiology · Pathology · Virulence genes · Uruguay

Introduction

Salmonella is an important pathogen worldwide that affects different animal species, including humans. The *Salmonella* genus comprises 2 species, *Salmonella bongori* and *Salmonella enterica.* This last species is made up of 6 subspecies with more than 2500 reported serotypes and is responsible for 93.8 million human cases of diarrhea and 155,000 deaths per year [1–3].

Circulating serotypes differ among geographic regions, although little information is available from South American countries. A systematic review that summarizes the serotypes reported in apparently healthy cattle throughout the world from 2000 to 2017 indicates that Montevideo, Typhimurium, Kentucky, Meleagridis, Anatum, Cerro, Mbandaka, Muenster, Newport, and Senftenberg were the most frequent. *Salmonella* Montevideo was the most

Responsible Editor: Luiz Henrique Rosa

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frequent in North America, *S*. Dublin in Europe, and *S*. Typhimurium in Africa, Asia, and Australasia [4]. Surprisingly, with regard to South American countries, this review included information from only one Venezuelan study and claimed that Javiana and Weltevreden were the only serotypes identified, although this information is not readily available in the original reference [5].

In a recent study involving 50 dairy farms in Argentina, *S. enterica* was detected in 36% of the farms, and the most prevalent serotypes were Mbandaka, Anatum, Typhimurium, Dublin, and Montevideo [6]. In Uruguay, *S.* Typhimurium was the most frequently isolated serotype in a study performed in 30 dairy farms with spontaneous outbreaks of neonatal calf diarrhea, while the serotype Anatum was isolated in a small subset of diarrheic and healthy calves [7]. In this country, the Dublin serotype was identified in calves with fatal septicemia [8], as well as in humans [9]. The identification of the bovine-adapted serotype Dublin in invasive infections in humans in Uruguay [9] raises concerns about direct or indirect bovine-to-human transmission.

The clinical outcome and severity of salmonellosis in cattle depend on the age, physiologic status of the animals, infecting serotypes, and infective dose [10, 11]. *Salmonella* Dublin and Typhimurium frequently cause clinical disease in cattle, although various other serotypes, including Montevideo and Newport, can also be involved in other clinical presentations, such as abortion [12, 13]. In calves, these serotypes can cause morbidity higher than 50% with lethality up to 100% if no medical treatment is applied. The economic impact of salmonellosis is based on reduced productivity, animal deaths, diagnostic expenses, and treatments [12].

Bovine salmonellosis is frequent in intensive farming systems, and calves have a higher risk of infection. In calves, *S.* Typhimurium usually causes diarrhea, fever and, less frequently, septicemia. Calves infected with *S.* Dublin can show diarrhea, fever, septicemia, respiratory distress, dry gangrene in the legs, tail or pinnae, and sudden death [10, 14]. In pregnant cows and heifers, *S.* Dublin can cause abortion in the presence of pyrexia or without any other obvious clinical signs [15, 16].

After digestive transmission, *S. enterica* unfolds a battery of virulence attributes that lead to the invasion and colonization of many organ systems. After crossing the intestinal barrier, Salmonellae can colonize the liver and gallbladder, spleen, lungs, brain, urinary tract, and bone marrow. Macroscopic lesions can be absent, or there could be enteritis/typhlitis/colitis with petechiae in the submucosa and serosa of the intestine, mesenteric lymphadenomegaly, fibrinous peritonitis, splenomegaly, and hepatomegaly. The lungs can have congestion, edema, or embolic interstitial pneumonia with exudation of fibrin and neutrophils in the lumen of the alveoli [12, 17]. *Salmonella* Typhimurium frequently causes enteric lesions, including necrotizing enteritis/colitis with watery, bloody, or fibrinous, malodorous intestinal contents [10].

Clinical and pathological findings in cases of salmonellosis can be explained by the presence of genetic virulence determinants of the pathogen. Once in the host, these mechanisms are activated and allow bacterial survival and further replication in the tissues. Many of these mechanisms are encoded by genes in *Salmonella* pathogenicity islands (SPIs), which are involved in cellular invasion, survival, and replication inside phagocytic cells, and their protein products are responsible for the lesions and the clinical signs [18].

Here, we describe epidemiological data, clinical signs, and pathological findings in spontaneous outbreaks of salmonellosis in dairy farms in Uruguay and their virulence genes (VGs) in the causative *S. enterica* Typhimurium and Dublin isolates. Second, we explore possible associations among these variables.

Materials and methods

Epidemiological data and clinical signs

Fifteen outbreaks of salmonellosis with fatal outcomes in dairy calves were studied upon request from field veterinary practitioners at the Instituto Nacional de Investigación Agropecuaria (INIA) veterinary diagnostic laboratory from 2016 to 2018. All outbreaks occurred spontaneously at commercial dairy farms in Uruguay. The primary reason for consultation (herd-level health problem) indicated for each outbreak by the referring veterinarian was recorded. Epidemiological data (number of exposed calves, number of clinically affected calves, and number of deceased calves in the exposed group) were obtained and used to calculate the morbidity, mortality, and lethality risks for each outbreak. Additionally, the clinical signs that the affected calves subjected to autopsy (see below) showed before dying, as reported by the referring veterinarians, as well as their age in days, were collected in all cases.

Pathologic examination

Autopsies of 20 naturally deceased calves from all 15 outbreaks were performed, and the macroscopic lesions were recorded. Tissue samples from all autopsied carcasses were collected and immersion-fixed in 10% neutral buffered formalin (pH 7) for a minimum of 48 h and processed routinely for histology [19]. Briefly, tissues were dehydrated through immersion in a series of solutions of ethanol and xylol and embedded in paraffin wax (Histoplas, Sakura) in an automated vacuum infiltration tissue processor (Tissue-Tek VIP Jr., Sakura). Paraffin-embedded tissue blocks were made in

a tissue embedding console (Tissue-Tek TEC 5, Sakura) and then sectioned at 4–5 µm in a rotatory microtome (Accu-Cut SRM 200, Sakura). Tissue sections were mounted on glass slides and stained with hematoxylin and eosin (Sigma Aldrich) in an automated slide stainer (Tissue-Tek Prisma, Sakura). Slides were examined under an optic microscope (Axio Scope. A1, Carl Zeiss) by an anatomic veterinary pathologist, and microscopic lesions were recorded.

Salmonella enterica isolate sources and serotyping

Selected tissue or fluid samples from all autopsied calves were collected aseptically at autopsy and subjected to selective culture for *S. enterica*, as described by Casaux et al., 2019. A total of 20 *S. enterica* isolates, one from each autopsied calf, were serotyped following the Kauffman-White-LeMinor scheme [20] at the bacteriology service of the "Instituto de Higiene, Universidad de la República (UdelaR)" in Montevideo, Uruguay.

Virulence gene detection

All *S. enterica* isolates were previously stored (− 80 °C) in trypticase soy agar (TSA)-glycerol (20%), grown in TSA (24 h, 37 °C), and later analyzed for the detection of 17 virulence genes by PCR. After incubation, two colonies of a pure culture were picked and suspended in 100 µl of distilled water. DNA extraction was conducted by boiling the suspension for 15 min, followed by centrifugation, and the supernatant was stored at − 20 °C. The presence of the virulence gene *spv*B associated with growth within the host; genes *spi*A, *pag*C, and *msg*A related to survival within macrophages; genes *inv*A, *prg*H, *org*A, *tol*C, *lpf*C, *sop*B, and *pef*A related to host recognition/invasion; genes *iro*N and *sit*C, the latter as a representative of the *sit*ABCD operon and both responsible for iron acquisition; gene *sif*A associated with filamentous structure formation; and genes *spa*N and *sip*B linked to entry into non-phagocytic cells and killing of macrophages, were assessed by PCR following procedures and primers described by Skyberg et al. [21]. The *stn* gene, which is responsible for the enterotoxicity of *Salmonella*, was amplified according to Murugkar et al. [22] using primers described by Gharieb et al. [23].

The amplification reactions were performed in a ProFlex PCR System (Applied Biosystems, Weiterstadt, Germany), and PCR products were run in 1% agarose gels stained with GoodView (SBS, China). To confirm their identities, representative amplicons for every gene were purified and sequenced at Macrogen Inc. (Seoul, South Korea) using the same primers used for PCR. The obtained sequences were compared with those in the database of the "National Center for Biotechnology Information" (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLASTN tool [24] and later used as positive controls for PCR. A tube containing the PCR mix without DNA addition was used as a blank for every reaction.

Statistical analyses

The available information was used to conduct exploratory statistical analyses, despite the small sample size. Reasons for consultation and epidemiological data (morbidity, mortality, and lethality) for all 15 outbreaks were recorded. Similarly, the age, clinical signs, and gross and microscopic pathological findings for each of the 20 calves, as well as the serotypes and virulence genes for all *S. enterica* isolates, were stored in LibreOffice Calc (Supplementary Table 1) and later analyzed in R software (R Foundation for Statistical Computing, Vienna, 2011, version 3.6.2). Variables with < 20% variability, such as the occurrence of the clinical sign coughing/tachypnea, some macroscopic lesions (those involving the rumen, urinary bladder, and brain/rete mirabile), microscopic lesions (enteritis and hepatitis), and most virulence genes (all except for *iro*N and *pef*A), were excluded from posterior analyses.

Pearson's chi-square test was implemented to assess the association between the VGs as well as between serotypes, clinical signs, pathological findings, and virulence genes. The permutation test was used as an approximation of Fisher's exact test to address the small sample size. Additionally, to test the hypotheses of relationships between epidemiological data (morbidity, mortality, and lethality), the reasons for consultation, age, clinical signs, pathological findings, the involved *S. enterica* serovars, the occurrence of virulence genes, and generalized linear regression models were fitted, and odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated. For all tests, the results were regarded as significant if $p < 0.05$.

Results

Salmonella enterica serotypes, reasons for consultation, and epidemiological data

Information on the sample of origin, the *S. enterica* serotypes, and the age of each calf in each outbreak is summarized in Table 1. The number of exposed, sick, and deceased calves in each outbreak, as well as the calculated morbidity, mortality, and lethality risks, are shown in Table 2.

Digestive disease was the most common reason for consultation (8 outbreaks), followed by sudden death (4 outbreaks), depression (2 outbreaks), and respiratory disease (1 outbreak) (Table 2). Seventeen calves from 11 outbreaks were infected with *S.* Typhimurium, and 5 calves from 4 outbreaks were infected with *S.* Dublin (Tables 1 and 2).

Table 1 Sample of origin (source) and serotype of the *Salmonella enterica* isolates obtained from 20 deceased dairy calves in 15 spontaneous outbreaks of salmonellosis

Outbreak number	Serotype	Calf ID	Age (days)	Source
I	Typhimurium	1	14	Mesenteric lymph node
	Typhimurium	2	16	Intestinal content
II	Typhimurium	1	20	Mesenteric lymph node
	Typhimurium	2	20	Mesenteric lymph node
III	Typhimurium	1	15	Mesenteric lymph node
IV	Typhimurium	1	5	Mesenteric lymph node
V	Typhimurium	1	10	Mesenteric lymph node
	Typhimurium	1	45	Mesenteric lymph node
VII	Typhimurium	1	10	Mesenteric lymph node
VIII	Typhimurium	1	8	Mesenteric lymph node
IX	Typhimurium	1	90	Intestine
X	Typhimurium	1	15	Mesenteric lymph node
XI	Dublin	1	50	Lung
	Dublin	2	50	Mesenteric lymph node
XII	Dublin	1	64	Mesenteric lymph node
XIII	Typhimurium	1	30	Mesenteric lymph node
	Typhimurium	2	30	Liver
	Typhimurium	3	30	Mesenteric lymph node
XIV	Dublin	1	60	Mesenteric lymph node
XV	Dublin	1	60	Kidney

All 8 outbreaks with digestive disease as the main reason for consultation were caused by *S.* Typhimurium, while 3 of the 4 outbreaks with sudden death as the main reason for consultation were caused by *S.* Dublin. In the generalized linear regression model, the serotype Dublin was a significant predictor of sudden death as a reason for consultation (OR = 30, 95% CI 2–1282, $p = 0.03$).

For the two most frequent reasons for consultation (digestive disease and sudden death), the average morbidity and mortality rates were 51.67% (range: 10–100%) and 25.4% (range: 3.29–78.9%) for digestive disease ($n = 8$ outbreaks) and 17.76% (range: 3.29–30%) and 17.32% (range: 1.97–30%) for sudden death ($n = 3$ outbreaks, no data = 1 outbreak) (Table 2). The average lethality risk for outbreaks with digestive disease as the reason for consultation was 51.4% (range: 10–100%) and 86.67% (range: 60–100%) for sudden death. The average morbidity and mortality risks for the outbreaks involving the Typhimurium ($n = 11$ outbreaks) and Dublin ($n = 3$ outbreaks, missing data = 1 outbreak) serotypes were 42.35% (range: 10–100%) and 23.02% (range: 3.85–78.95%) for the Typhimurium serotype and 9.35% (range: 3.29–20%) and 8.46% (range: 1.97–20%) for the Dublin serotype, respectively. The average lethality risk for the outbreaks involving the serotypes Typhimurium and Dublin was 62.38% (range: 10–100%) and 77.14% (range: 71.43–100%), respectively. Morbidity, mortality, and lethality risks did not differ significantly among these reasons for consultations or serotypes.

Clinical signs, age, and serotypes

The clinical signs observed in the autopsied calves before death and reported by the veterinarians included diarrhea (14 cases), neurological signs/depression (4 cases), and coughing/tachypnea (3 cases). Two calves had diarrhea and neurological signs/depression (1 for each serotype), and 1 calf (infected with *S.* Typhimurium) had diarrhea and coughing/tachypnea. The most common clinical sign in the15 cases infected with *S.* Typhimurium was diarrhea (13 cases), followed by neurological signs/depression (1 case) and coughing/tachypnea (1 case), while the 5 calves infected with the Dublin serotype most frequently manifested neurological signs/depression (2 cases), followed by coughing/tachypnea (1 case) and diarrhea (1 case). One of the *S.* Dublin-infected calves was reported to have died suddenly without observable clinical signs.

The average age of the 5 calves affected by *S.* Dublin was 55 days (min. 50 d, max. 60 d), while 15 calves affected by *S.* Typhimurium had an average age of 23.9 d (min. 5 d, max. 90 d; Table 1); this difference was statistically significant (Fig. 1). In the generalized linear regression model, age was a significant predictor of the infecting serotype; for each one-day increment in age, the odds of *S.* Typhimurium (vs. *S.* Dublin) infection decreased by 0.1 (OR = 0.9, 95% CI 0.8–1, $p = 0.03$).

The average age of the 14 calves that had diarrhea as a clinical sign before death was 22.4 days, while the 6 calves that did not have diarrhea averaged 53.3 days; this difference was statistically significant (Fig. 2). Similarly, in the generalized linear regression model, age was a predictor of diarrhea; for each one-day increment in age, the odds of diarrhea decreased by 0.08 (OR = 0.92, 95% CI 0.8–1, $p = 0.02$). In calves with diarrhea, the age was 60 days for the single case infected with the Dublin serotype and averaged 19.46 days for the calves infected with *S.* Typhimurium.

Table 2 Reasons for consultation and epidemiological data in 15 outbreaks of salmonellosis in dairy calves

Reason for consultation		Serotype	Number of exposed calves	Number of sick calves	Morbidity (%)	Number of dead calves	Mortality (%)	Lethality (%)
Digestive disease	Outbreak **I**	Typhimurium	20	2	10.0	2	10.0	100
	Outbreak **II**	Typhimurium	5	4	80.0	2	40.0	50.0
	Outbreak **III**	Typhimurium	26	10	38.5	1	3.8	10.0
	Outbreak **IV**	Typhimurium	41	11	26.8	3	7.3	27.3
	Outbreak **V**	Typhimurium	20	4	20.0	1	5.0	25
	Outbreak **VI**	Typhimurium	19	19	100.0	15	78.9	78.9
	Outbreak **VII**	Typhimurium	5	5	100.0	1	20.0	20.0
	Outbreak **VIII**	Typhimurium	42	16	38.1	16	38.1	100
Respiratory disease	Outbreak **IX**	Typhimurium	200	20	10.0	15	7.5	75.0
Depression	Outbreak **X**	Typhimurium	40	5	12.5	5	12.5	100
	Outbreak **XI**	Dublin	147	7	4.8	5	3.4	71.4
Sudden death	Outbreak **XII**	Dublin	152	5	3.29	3	1.97	60.0
	Outbreak **XIII**	Typhimurium	50	15	30.0	15	30.0	100
	Outbreak L **XIV**	Dublin	100	20	20.0	20	20.0	100
	Outbreak **XV**	Dublin	ND	ND	-	ND	-	-

[A]ND: No data

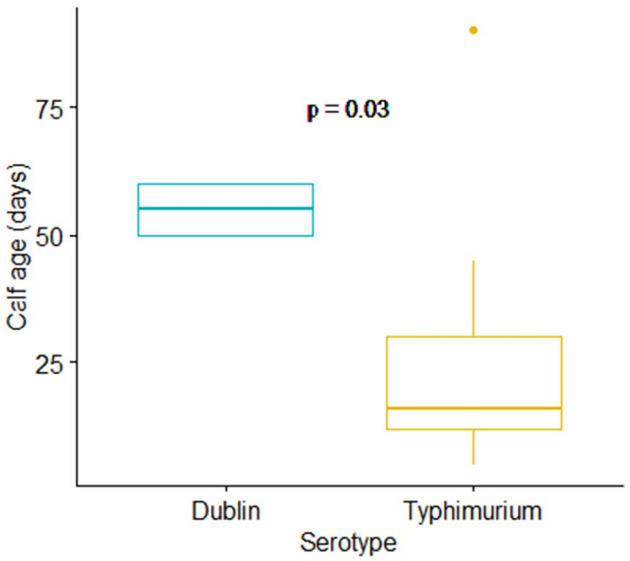

Fig. 1 Boxplot distribution of the age of the calves with salmonellosis caused by *S.* Dublin (average = 55 days, $n = 5$ calves) and *S.* Typhimurium (average = 23.9 days, $n = 15$ calves)

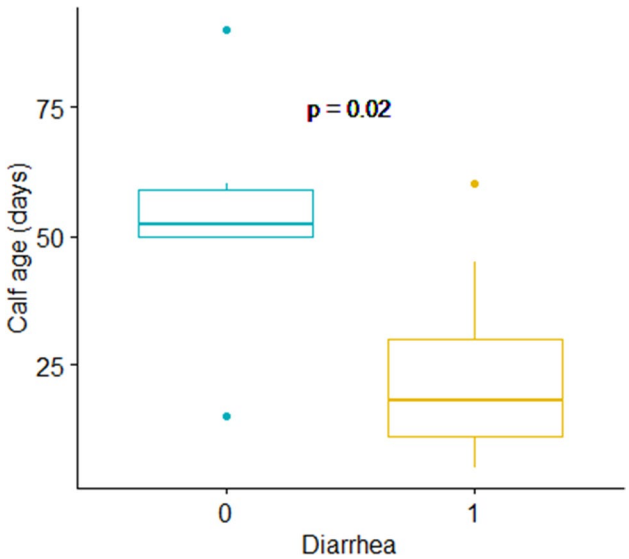

Fig. 2 Boxplot distribution of the age of the calves with salmonellosis without (average = 53.3 days, $n = 6$, left plot) or with (average = 22.4, $n = 14$ calves, right plot) manifestation of diarrhea as a clinical sign before death

Macroscopic and microscopic lesions

Gross lesions were recorded in 20 autopsied calves (Table 3; Fig. 3). The anatomic location of the lesions included serosae or joints (11/17), the intestinal tract (10/20), the liver (7/18), the adrenal glands (6/13), the lungs (6/16), the mesenteric lymph nodes (8/10), the spleen (5/16), the abomasum (4/16), and the cerebral meninges (1/16). No gross lesions were identified in the heart or kidneys. One calf (number 2 of outbreak XI) had jaundice and fibrinous cholecystitis.

All 10 calves with macroscopic lesions in the intestinal tract were infected with the Typhimurium serotype. None of the 3 calves infected with the Dublin serotype for which there were data on gross examination of the intestinal tract had grossly visible enteric lesions.

Table 3 Macroscopic lesions in 20 calves with salmonellosis subjected to autopsy

Outbreak	Animal ID, serotype[a]	Enteritis[b]	Hepatitis[c]	Adrenocortical hemorrhage	Pneumonia[d]	Mesenteric lymph node [e]	Splenomegaly	Abomasum[f]	Rumenitis	Urinary bladder [g]	Serositis or arthritis[h]	Brain[i]
I	**1, T**	+	+, A, B, C	+	+	+, B	-	-	-	-	+, B	-
	2, T	+	+, A, B	+	-	ND	-	-	-	-	+, A	-
II	**1, T**	-	-	+	+	ND	+	-	-	ND	+, C	-
	2, T	+	-	ND	-	ND	-	+, A	-	ND	+, C	-
III	**1, T**	-	-	ND	ND	+, A	-	-	+	-	-	-
IV	**1, T**	+	+, A, B, C, D	-	+	+, A	+	-	-	+, A, B	+, E	-
V	**1, T**	+	-	-	-	+, A	-	-	-	-	-	-
VI	**1, T**	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
VII	**1, T**	+	+, B	-	-	+, A	+	+, B	ND	-	+, B	-
VIII	**1, T**	ND	ND	ND	ND	+, A	ND	ND	ND	ND	ND	ND
IX	**1, T**	+	-	ND	-	+, A	-	-	ND	-	-	ND
IX	**1, T**	-	-	-	-	-	ND	-	-	-	+, E	-
XI	**1, D**	-	-	+	-	ND	+	-	-	ND	+, E	-
	2, D	-	+, B, C, D	+	+	+, A	+	ND	-	+, A	+, B	-
XII	**1, D**	ND	+, A, B, D	ND	+	ND	-	+, A, B	ND	ND	+B, C, E	+
XIII	**1, T**	+	-	+	-	ND	-	+, B	-	-	-	-
	2, T	+	-	-	-	ND	-	-	-	-	-	-
	3, T	+	-	-	-	ND	-	-	-	-	-	-
XIV	**1, D**	-	-	-	+	-	-	-	-	-	+, E	-
XV	**1, D**	ND	+, A	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a]*T*, Typhimurium; *D*, Dublin. [b]Includes fibrinous, erosive/ulcerative enteritis, colitis, typhlitis, or combinations (enterocolitis, enterotyphlitis, typhlocolitis, or enterotyphlocolitis). [c]Includes hepatomegaly (A) with or without diffuse ochre/yellowish discoloration of the parenchyma (cholestasis, B), with or without multifocal disseminated hepatitis (C), with or without fibrinous cholecystitis (D). [d]Includes multifocal disseminated embolic interstitial pneumonia and/or pulmonary edema. [e]Includes lymphadenomegaly (A) and/or fibrinosuppurative lymphadenitis (B). [f]Includes ulcerative abomasitis (A) or abomasal edema (B). [g]Includes petechiae on the mucosa (A) and/or fibrin in the urine (fibrinous urocystitis, B). [h]Includes fibrinous arthritis (A), fibrinous peritonitis (B), fibrinous pericarditis (C), fibrinous pleuritis (D), or serosal petechiae (E). [i]Includes congestion, edema, and cloudy cerebrospinal fluid. *ND*, not determined

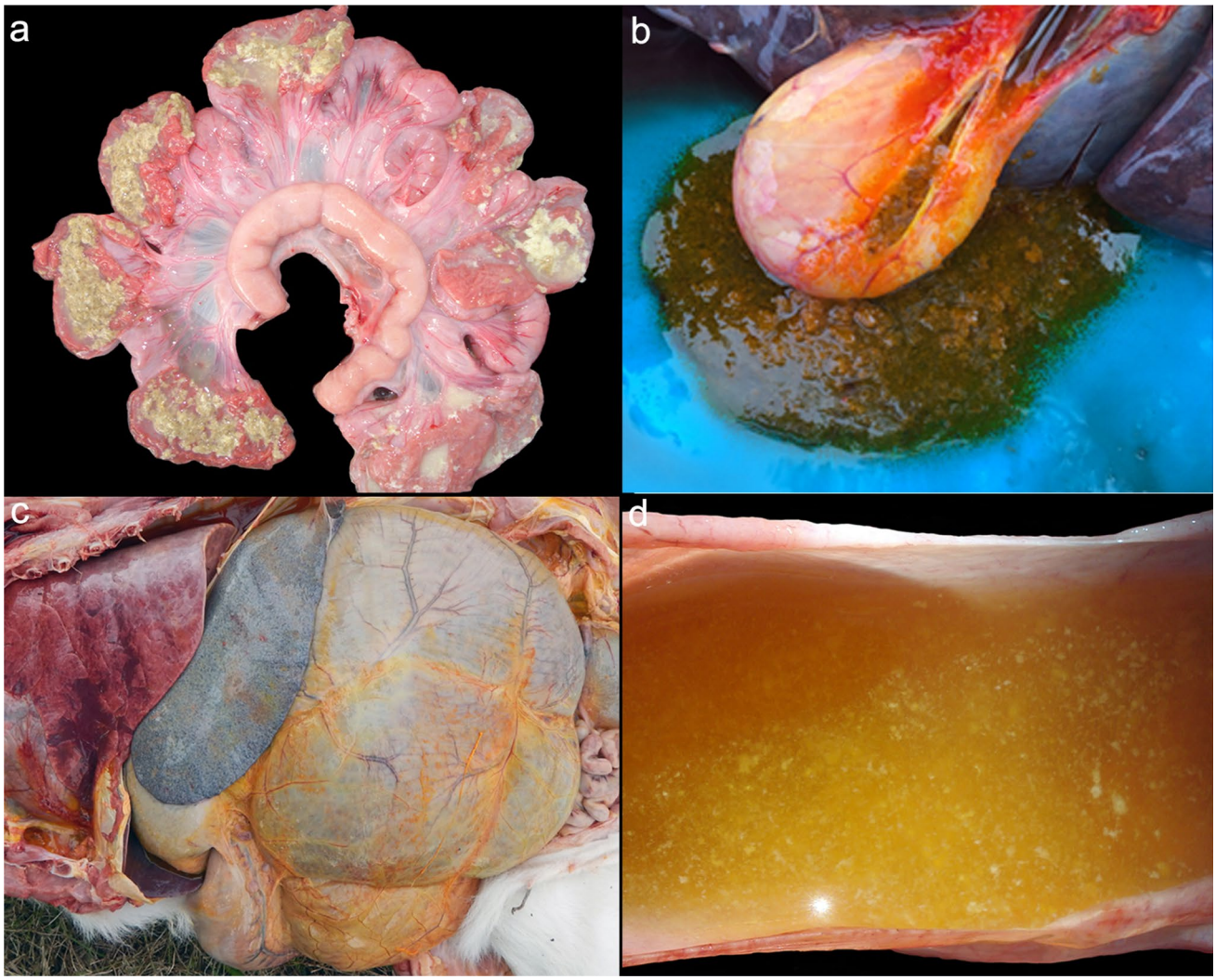

Fig. 3 Gross lesions in dairy calves with salmonellosis. (**a**) Calf 1, outbreak XIII. Segment of jejunum with abundant yellowish fibrillar material (fibrin) adhered to the mucosa (severe fibrinous enteritis); the mesenteric lymph nodes are enlarged and swollen (mesenteric lymphadenitis). (**b**) Calf 2, outbreak XI. The gallbladder is distended and contains bile admixed with abundant fibrin (fibrinous cholecystitis). (**c**) Calf 2, outbreak XI. Left side view of abdominal and thoracic organs in situ. The spleen is enlarged (splenomegaly), and there is yellow discoloration of the omentum, serosal surfaces of the forestomachs and adipose tissue (jaundice). The lung failed to collapse and showed rib imprints on the pleural surface and dark red discoloration of multiple lobules (interstitial pneumonia). (**d**) Calf 1, outbreak IV. The urinary bladder contains abundant urine with granular to fibrillar yellowish clots (fibrinous urocystitis)

The results of the histopathologic examination of various tissues of the 20 carcasses subjected to autopsy are shown in Table 4. The most consistent microscopic lesions were necrotizing enteritis in 19 of 19 calves, multi-focal random necrotizing and/or portal hepatitis in 18 of 19 cases, interstitial pneumonia in 13 of 17 cases, lymphadenitis in 12 of 15 cases, splenitis in 7 of 17 cases, abomasitis in 6 of 8 cases, adrenocortical hemorrhage in 7 of 14 cases, tubulointerstitial nephritis in 5 of 18 cases, and meningitis or perivasculitis in the rete mirabile in 4 of 15 cases. Selected microscopic lesions are shown in Fig. 4. There were no cardiac lesions observed. Splenitis was more frequent in calves infected with *S*. Dublin (4/5) than in calves infected with *S*. Typhimurium (2/13), while tubulointerstitial nephritis affected 4/5 of calves infected with *S*. Dublin and 1/13 of calves infected with *S*. Typhimurium.

Detection of virulence genes

There was < 20% variability in the detected virulence genes across isolates, except for the *pef*A and *iro*N genes. The absolute and relative frequencies for each of the 17 virulence genes detected in all *S*. Typhimurium and *S*. Dublin isolates are shown in Table 5. Eleven virulence genes (*inv*A, *sit*C, *org*A, *lpf*C, *sip*B, *msg*A, *sif*A, *tol*C, *sop*B, *prg*H, and *stn*) were detected in all *S*. Dublin isolates, while the *spa*N,

Table 4 Microscopic lesions in 20 calves with salmonellosis subjected to autopsy

Outbreak	Animal ID, Serotype[a]	Enteritis[b]	Hepatitis[c]	Adrenocortical hemorrhage	Pneumonia[d]	Fibrinous/suppurative lymphadenitis	Fibrinous/suppurative splenitis	Abomasitis[e]	Rumenitis/reticulitis	Urinary bladder[f]	Kidney[g]	Brain or rete mirabile[h]
I	1, T	+, A, B	+, A, C	+	+, A	+	-	+, A, B	ND	ND	-	-
	2, T	+, A, B	+, B, C	+	+, A	+	-	ND	ND	ND	-	-
II	1, T	+, A, B	+, B	+	+, A	-	-	+, A, B	-	-	-	-
	2, T	+, A, B	+, B	-	+, A, B	-	-	ND	+	ND	-	-
III	1, T	+, A, B	+, B	-	+, A, C	ND	-	ND	+	ND	-	+, B
IV	1, T	+, A, B, C	+, A	-	+, A, B	+	+	-	ND	+, A	-	-
V	1, T	+, A, B	+, A	ND	-	+	-	ND	-	-	-	ND
VI	1, T	+, A, B	+, B, C	ND	ND	+	ND	ND	ND	ND	ND	ND
VII	1, T	+, A, B	+, A, B	ND	-	+	+	ND	ND	ND	-	-
VIII	1, T	+, A, B, C	+, A	ND	ND	+	ND	ND	ND	ND	ND	ND
IX	1, T	+, A, B	+, A, B, C	-	+, A, B, C	+	-	+, A	-	-	-	-
X	1, T	+, A, B, C	+, B	-	+, A	ND	+	ND	ND	ND	-	-
XI	1, D	+, A, B	ND	+	+, A, B, C	+	+	-	-	+, B	-	+, B
	2, D	+, A, B	+, A	+	+, A, B, C	ND	+	ND	-	+, A	+	ND
XII	1, D	+, A, B, C	+, A, B, C	-	+, A, B, C	+	+	+, A, B	-	ND	+	+, B
XIII	1, T	+, A, B, C	-	+	-	ND	-	ND	ND	ND	+	-
	2, T	+, A, B, C	+, B	+	-	+	-	+, A, B	ND	ND	-	-
	3, T	+, A, B	+, B	ND	+, A	+	-	ND	ND	ND	-	-
XIV	1, D	+, A, B	+, A	-	+, A, B	-	+	+, A, B	-	ND	+	+, A
XV	1, D	ND	+, A, C	ND	ND	ND	ND	ND	ND	ND	+	ND

[a]*T*, Typhimurium; *D*, Dublin. [b]Includes fibrinous, suppurative, or fibrinosuppurative enteritis-colitis-typhlitis (A) or combinations thereof with epithelial necrosis (erosions/ulcers), necrotizing cryptitis (B), and microthrombosis of mucosal or submucosal blood vessels (C). [c]Includes focal or multifocal random neutrophilic/histiocytic necrotizing hepatitis (A), portal hepatitis (B), or cholestasis in the canaliculi or bile ducts (C). [d]Includes multifocal neutrophilic/histiocytic interstitial pneumonia (A), with alveolar edema or fibrinous/neutrophilic alveolitis/bronchiolitis (B), and microthrombosis in alveolar capillaries (C). [e]Includes inflammation in the lamina propria (A) with necrosis of the glandular or superficial epithelium (B). [f]Includes microhemorrhages in the mucosa/submucosa (A) and multifocal neutrophilic/histiocytic urocystitis (B). [g]Multifocal neutrophilic/histiocytic tubulointerstitial nephritis. [h]Includes meningitis (A) or fibrinous/neutrophilic perivasculitis in the rete mirabile (B). *ND*, not determined

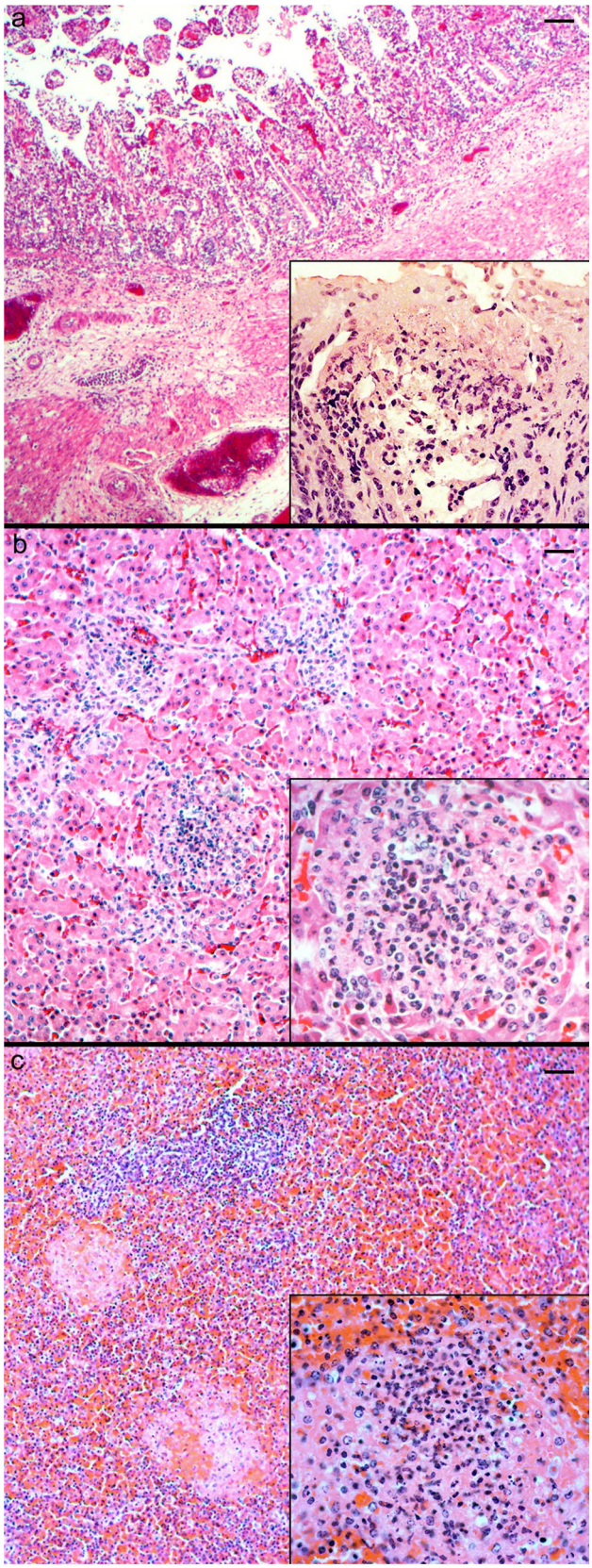

Fig. 4 Microscopic lesions in dairy calves with salmonellosis. (**a**) ▶ Calf 1, outbreak IV. The small intestinal histoarchitecture is effaced, and the lamina propria is expanded by degenerate neutrophils, proteinaceous material, and necrotic debris (inset). (**b**) Calf 1, outbreak IV. Multifocal random necrotizing hepatitis, characterized by infiltration of macrophages and neutrophils admixed with necrotic hepatocytes (inset). (**c**) Calf 1, outbreak XII. There is multifocal extravasation of fibrin entrapping macrophages and neutrophils (inset) in the red pulp of the spleen (splenitis). Hematoxylin and eosin stain. Bars = 100 µm

*sip*A, and *pag*C genes were detected in four isolates. The *spv*B gene was detected in 3 isolates, and *pef*A and *iro*N were detected in two isolates.

The genes *inv*A, *sit*C, *org*A, *sif*A, *sop*B, and *stn* were detected in 15 isolates of *S*. Typhimurium. Fourteen isolates had the *spv*B, *spa*N, *lpf*C, *sip*B, *msg*A, *pef*A, *tol*C, and *prg*H genes, while the *sip*A and *pag*C genes were present in 13 isolates. The gene *iro*N was present in only 2 isolates. Nine isolates of *S*. Typhimurium presented 16 genes, 5 isolates presented 15 genes, and 1 isolate presented 11 genes.

Analysis of virulence gene clinical signs, pathological findings, and epidemiological data

Pearson's chi-square tests were performed to determine the associations between the two virulence genes that had > 20% variability (*iro*N and *pef*A) among them and between serotypes, clinical signs, pathological findings, and these virulence genes. These two genes did not show a significant association between them. Regarding clinical signs, serotype, and gross lesions, the Typhimurium serotype was significantly associated with diarrhea ($p = 0.01$) and enteritis ($p = 0.03$), mesenteric lymphadenitis was associated with neurological signs ($p = 0.02$), and arthritis/serositis was associated with hepatitis ($p = 0.04$). When clinical signs, serotypes, and microscopic lesions (histology) were tested, the Dublin serotype was associated with tubulointerstitial nephritis ($p = 0.01$) and fibrinosuppurative splenitis ($p = 0.01$). For the clinical signs, serotype, and the two virulence genes subjected to analysis, the pefA gene was associated with the serotype Typhimurium ($p = 0.04$).

Regarding macroscopic and microscopic lesions, enteritis observed grossly was associated with microscopic meningitis or fibrinous/neutrophilic perivasculitis in the rete mirabile ($p = 0.03$), grossly visible arthritis/serositis ($p = 0.03$) and splenomegaly ($p = 0.04$) were associated with fibrinosuppurative splenitis, and gross and microscopic adrenal hemorrhage were associated ($p = 0.03$). Although microscopic enteritis and hepatitis were excluded from the statistical analysis due to low variability, all calves with grossly visible enteritis had microscopic enteritis, and all of the cases with grossly visible hepatitis had microscopic hepatitis, as expected. Regarding lesions and virulence genes, the *pef*A

Table 5 Frequency of 17 virulence genes detected in 20 isolates of *Salmonella enterica*

Gene	Function	*S*. Typhimurium	*S*. Dublin	Absolute frequency	Relative frequency (%)
*inv*A	Host recognition and invasion	15	5	20	100
*org*A		15	5	20	100
*prg*H		14	5	19	95
*tol*C		14	5	19	95
*sop*B		15	5	20	100
*lpf*C		14	5	19	95
*pef*A		14	2	16	80
*spa*N	Macrophage invasion	14	4	18	90.9
*sip*B		14	5	19	95
*iro*N	Iron acquisition	14	5	19	95
*sit*C		15	5	20	100
sifA	Filament formation	15	5	20	100
*pag*C	Intramacrophage survival	13	4	17	85
*msg*A		14	5	19	95
*spi*A		13	4	17	85
stn	*Salmonella* enterotoxin	15	5	20	100
spvB	Growth within host	14	3	17	85

gene was associated with macroscopic enteritis ($p = 0.03$) and with microscopic fibrinosuppurative splenitis ($p = 0.04$).

Univariable generalized linear regression analyses revealed that the Typhimurium serotype was associated with diarrhea (OR = 26, 95% CI 2–695, $p = 0.01$). The Dublin serotype was associated with tubulointerstitial nephritis (OR = 48, 95% CI = 3.5–1979, $p = 0.01$). Finally, the *pef*A gene was associated with the Typhimurium serotype (OR = 21, 95% CI 2–578, $p = 0.03$).

Discussion

Calf morbidity and mortality lead to significant productive and economic losses in dairy farms [25]. Morbidity and mortality values of 25% and 5%, respectively, have been regarded as acceptable in dairy calf rearing systems [26]. However, in Uruguay, the annual dairy calf mortality risk between birth and weaning is 15.2%, with neonatal diarrhea and respiratory disease being the main farmer-reported clinical signs shown by the calves before death [27].

Salmonella enterica is one of the main causative agents of enteritis, septicemia, and respiratory disease in rearing calves [28, 29]. In the 15 outbreaks of salmonellosis evaluated in this manuscript, the average morbidity, mortality, and lethality risks were 35.3%, 19.9%, and 65.6%, respectively, which exceed acceptable values and the average mortality risk reported in 225 dairy farms in Uruguay [27]. This finding is in concert with a previous study by our group in which enteric *S. enterica* infection was associated with an increased risk of mortality, especially in diarrheic calves [30], and provides evidence for the virulence of the acting strains. Morbidity, mortality, and lethality risks did not differ significantly in outbreaks caused by either serotype in our study.

The clinical signs of salmonellosis in calves can vary from diarrhea, fever, loss of appetite, respiratory signs, encephalitis, swollen joints, gangrene of the limbs, or tips of the pinnae, to decreased production (reduced weight gain or weight loss) [31]. In this document, the reported clinical signs were mainly diarrhea and, to a lesser extent, respiratory and nervous signs. However, in Uruguay, health data recording in dairy farms is generally scarce [27], and therefore, there might have been other unreported signs. Our results indicate that when digestive disease or diarrhea are the reasons for consultation or the main clinical sign shown by the calves before dying, *S*. Typhimurium is the most likely serotype involved, especially in younger calves. Possibly, the adaptations of the digestive tract of neonates and the action of this serotype favor this presentation. The Dublin serotype was detected in 5 calves of 50 to 60 days of age from 4 outbreaks in which sudden death was the main reason for consultation. Recently, septicemic salmonellosis caused by *S*. Dublin in 1- to 3-month-old calves was described in Uruguay and Brazil [8, 32, 33]. On the other hand, in the pathogenesis of digestive disease caused by Typhimurium serovar, animals show an enteric inflammatory process that ultimately leads to diarrhea [34]. In this study, 13 cases of 9 outbreaks in which the Typhimurium serotype was identified presented diarrhea as the main clinical manifestation. However, septicemic salmonellosis was caused by both serotypes.

In the present study, the majority of macroscopic lesions observed at autopsy were highly suggestive of salmonellosis. In *S. enterica*-related outbreaks, macroscopic changes are observed mainly in the intestine, mesenteric lymph nodes, liver/gallbladder, spleen, and lungs, although lesions are not necessarily restricted to these organs [12]. In our study, the presence of enteritis grossly was associated with the Typhimurium serotype, and thus, finding this lesion upon autopsy should raise a suspicion of *S*. Typhimurium, especially in younger calves with diarrhea. In the present study, lesions were frequent in the mesenteric lymph nodes, liver, and lungs, while isolates were obtained from the same tissues and the intestinal contents. Sporadically, the kidneys, gall bladder/bile, spleen, cecum, ileum, and urinary bladder were also affected. By virtue of the main lesions found in our study and by Pecoraro et al. [17], we suggest that liver, mesenteric lymph node, ileum, cecum, bile, and lung samples are the most important for successful bacteriological culture. Similar samples were proposed by Holschbach and Peek [31]. In accordance with Guizelini et al. [32] and Ramos et al. [35], the main macroscopic lesions observed in our work were enteritis with or without fibrin, mesenteric lymphadenomegaly, and hepatomegaly. In contrast to what was reported by Pecoraro et al. [17], in our work, macroscopic lesions in the lungs and spleen were less frequent. Additionally, in all *S*. Dublin cases, macroscopic intestinal lesions were absent, and calves did not show diarrhea except for one case. Similar results were observed by Guizelini et al. [32] in Brazil. Assays performed with this serotype have shown that the aflagellated types isolated from the bloodstream can generate a lower intestinal inflammatory response and increased invasiveness [9].

Upon histologic examination, we observed enterocolitis, enterotyphlitis, typhlocolitis, or enterotyphlocolitis with necrotizing cryptitis as the most frequent lesion in calves infected with either serotype. Inflammatory activity and cytokine release at the intestinal level increase the process of cell injury, dehydration, and diarrhea that can, ultimately, lead to the death of the affected animals [31, 36]. These processes demonstrate the first contact of the bacteria with the tissues and the neutrophilic infiltrate, the response for preventing its spread. At a microscopic level, we confirmed the systemic process by detecting random necrosis in the liver and/or portal hepatitis, interstitial pneumonia, fibrinosuppurative splenitis and mesenteric lymphadenitis, and/or meningitis or inflammatory lesions in the rete mirabile. We also observed adrenocortical hemorrhage for both *S*. Dublin and *S*. Typhimurium serotypes, which is generally associated with acute infectious processes and circulatory imbalances and is often referred to as Waterhouse-Friderichsen syndrome [37]. The bacteria reach the adrenal gland, affecting the blood vessels with the consequent hemorrhage and failure of the gland [38]. We observed ulcerative abomasitis with abomasal edema. Abomasitis is a clinical syndrome that is present in lactating calves with the possible involvement of species of *Clostridium*, including the former *Sarcina* sp. and *S*. Typhimurium DT104 [39, 40], but in our work, none of our strains were DT104 (data not shown). However, to date, the implications of these agents remain unknown, and a multifactorial etiology has been postulated [41]. Hemorrhages in the urinary bladder, urocystitis, and tubulointerstitial nephritis have been described occasionally in calves that died from *S*. Dublin septicemia [8]. In our current work, *S*. Dublin was significantly associated with tubulointerstitial nephritis and fibrinosuppurative splenitis. These lesions, especially in older age with sudden death, should raise a suspicion for the Dublin serotype.

The molecular mechanisms by which *Salmonella* produces lesions have been studied [18, 42, 43]. Depending upon the stage of pathogenesis, different genetic mechanisms are involved in intestinal and systemic disease. Various environmental mechanisms activate and upregulate SPI [44] to achieve colonization, invasion, and spread into the host [45]. The pathogenicity islands SPI-1 and SPI-2 encode a type III secretion system (TTSS) that translocates effector proteins into host cells [46]. In general, SPI-1 genes are activated in the initial contact of the bacterium with host cells and are responsible for cell invasion, host immune and inflammatory responses, recruitment and apoptosis of immune cells, and the formation of biofilms [46, 47]. This study is the first to describe the frequency of a relatively large set of 17 virulence genes in *S*. Dublin and *S*. Typhimurium, isolated from fatal disease in cattle. These strains present vast virulence mechanisms to act in the different stages of infection. We detected a high frequency of the *inv*A, *prg*H, *org*A *spa*N, and *sip*B genes involved in the type III secretion complex, which are carried by most of the isolates studied from chickens, turkeys, and pigs [21, 48]. Most of these genes encode structural proteins that take part in channel formation and protein exportation [46, 49], which in the end allow the effector proteins of the secretion system to enter the cytosol [50].

On the other hand, as previously indicated, enteric lesions were one of the most frequent findings in our study. Many of the SPI-1-encoded proteins act on the host inflammatory response by inhibiting macrophage proinflammatory cytokine expression and leukocyte activity and inducing diarrhea [51]. The *sip*B gene was detected at high frequency. This gene is necessary to induce caspase-1-dependent macrophage pyroptosis and interleukin-18 release [52–54]. In addition, we detected a high frequency of *sop*B, a gene that promotes the secretion of fluids and chloride, induces inflammation and diarrhea, acts on polymorphonuclear cells [55–58], has antiapoptotic activity, and is related to intracellular replication [59] and damage to the epithelial barrier during intestinal invasion [60].

We also found the *tol*C gene in high frequency. This gene is part of an efflux system called AcrAB-TolC, which is necessary for the adhesion and invasion of epithelial cells. Furthermore, it confers innate resistance to a wide range of toxic substances, including tetracyclines [61] and fluoroquinolones, artificial and natural colorants, disinfectants, and detergents such as bile. When evaluating the antimicrobial resistance of the strains included in this work, we detected resistance to tetracycline and intermediate resistance to ciprofloxacin in 80% and 55% of the isolates, respectively [7]. In this context, in which efflux pumps participate in virulence through *Salmonella* adhesion and invasion, a more in-depth study is warranted to detect the implication of this mechanism in resistance to certain antibiotics.

The *lpf*C and *pef*A genes act in the first phase of contact and union of the bacterium with the intestine [49]. The *lpf*C gene, along with the bovine colonization factor (Bcf) necessary for colonization of bovine Peyer's patches, is required for long-term intestinal persistence of *S*. Typhimurium [62]. Accordingly, it would be appropriate to evaluate the presence of the *bcf* gene and determine if it could explain disease recurrence.

In the second stage of the infection process, the genes present in SPI-2 are expressed. The *sif*A gene stabilizes the *Salmonella*-containing vacuole (SCV) membrane, along with SPI-2 genes and persistent gene products in the cell cytosol derived from SPI-1, triggers intracellular events, and together contributes to establishing a niche of *Salmonella* in macrophages [49]. Associated with this mechanism, *pag*C and *msg*A are encoded in small islets of pathogenicity [63], detected together with *spi*A in 85% to 95% of our isolates. Additionally, the *spi*A gene encoded by SPI-2 regulates the secretion of proteins translocated under conditions that simulate the vacuolar environment and interferes with gallbladder trafficking, intracellular bacterial proliferation, and secretion [49].

Siderophores are bacterial molecules that carry iron and are important for bacterial growth in serum in the extracellular stage of systemic infection. *Salmonella* produces two siderophores, salmochelin and enterobactin. Salmochelin requires two genetic loci for a correct function, and the *fep* gene group and the iro operon comprise five genes. The *iro*N gene is one of the last to be expressed and encodes a membrane receptor. In this work, we found a high frequency of the *iro*N gene. Regardless of the presence of this gene, bacteria can acquire nutrients through various mechanisms. One of them is the *sit*ABCD operon, present in every isolate of our collection that is induced in iron-deficient conditions after invasion of the intestinal epithelium [64, 65]. The iron operon sit system might be the mechanism that allows *Salmonella* to overcome iron deficiency and establish an infection. The *spv*B gene, found in a very transmissible plasmid in *Salmonella*, was detected in almost every isolate of this work. Its presence is associated with the inhibition of autophagy, a mechanism of the immune system that is frequently used by the host to eliminate the pathogen [66]. The proteins they encode are translocated to the cell by the TTSS of SPI-2. The protein that encodes *spv*B prevents actin polymerization, destabilizes the cytoskeleton of infected cells, modulates the maturation and positioning of SCV, and induces apoptosis [67]. The plasmid carries four highly conserved genes (ABCDs) and correlates with greater virulence of the strains [68, 69].

The *stn* gene was detected in every isolate of the collection, as reported before. Widely distributed in *Salmonella*, it has been proposed as a target for diagnosis [70]. It acts in the early stages of pathogenicity of bacteria, causing diarrhea [71], and helps maintain the composition and integrity of the membrane [72]. Despite these facts, there is still no agreement on its role in virulence.

Overall, the strains of the two serotypes we studied have similar virulence genes that fulfill different structural or effector functions. In this sense, it could be inferred that the strains that affected the calves could belong to a common strain of *Salmonella* circulating in the study region, although further research is needed to elucidate this possibility. The use of new techniques such as single nucleotide polymorphisms (SNPs) has allowed the detection of genetic variants associated with diseases. Currently, their importance is widely recognized and justifies applying these molecular techniques to the study of virulence genes present in strains [73].

As previously described, in addition to observing a high frequency of diarrhea, we observed a significant association between *S*. Typhimurium with diarrhea and enteritis and the *pef*A gene and the Typhimurium serotype. This gene is located in plasmids [49]. The association between the Typhimurium serotype and the *pef*A gene could explain the high frequency of diarrhea in the presence of this serotype since this gene allows bacterial adhesion to intestinal epithelial cells. In this work, only 2/5 isolates of *S*. Dublin obtained from 5 calves had the *pef*A gene. This finding could be due to the presence of plasmids that carry the gene.

Both serotypes can cause systemic infection. In this work, we identified an association between mesenteric lymphadenitis and neurological signs and between gross enteritis and microscopic meningitis or fibrinous/neutrophilic perivasculitis in the rete mirabile, which indicates that *Salmonella* can spread from an intestinal location and alter the integrity of the blood–brain barrier, presumably through inflammatory mechanisms [74]. In this work, arthritis/serositis was associated with hepatitis and splenomegaly with fibrinosuppurative splenitis. Cytokine production in bacterial infections is rigorously balanced [75]. Th17 lymphocytes present in intestinal inflammation due to *Salmonella* in humans [76] play an important role

in the development of reactive arthritis due to the production of interleukin 17, which has receptors at the joint level [77–79]. On the other hand, the deficiency of this interleukin is associated with the production of granulomas in different organs due to intracellular pathogens such as *Salmonella*, and its downregulation was associated with the absence of sepsis and lesions [73]. These associations could explain the findings of this work. Immune reactions and their balance play an important role in *Salmonella* infection. Considering the breadth of its function, it would be appropriate to evaluate its behavior in a bovine model.

In our study, as age increased, calves were less likely to present diarrhea and be infected with *S*. Typhimurium, which could be related to the immunological development of calves, as well as the physiological development of the rumen, the establishment of microorganisms in the rumen and intestine and the reduction in abomasal pH [80]. In addition, as age increased, calves were more likely to be infected with *S*. Dublin. Segall and Lindberg (1991) [81] suggested that this finding could be linked to factors that develop in calves over time, but there are no studies to date that explain this finding, and more investigation is needed.

Conclusions

Salmonellosis represents an important cause of death in dairy calves in Uruguay. In 15 outbreaks evaluated due to *S*. Dublin and *S*. Typhimurium, the epidemiological data on morbidity, mortality, and lethality obtained far exceed acceptable production values. Diarrhea followed by respiratory and nervous signs were the main clinical manifestations. The virulence genes repertoire was similar in *S*. Dublin and *S*. Typhimurium, most genes, but *pef*A and *spv*B, were shared in both. *Salmonella* Typhimurium was observed to be related to the manifestation of diarrhea and associated with macroscopic enteritis and the *pef*A gene in calves less than 30 days old and *S*. Dublin with sudden death in calves at approximately 50 days, mostly without intestinal lesions. Microscopically, enterocolitis, enterotyphlitis, typhlocolitis, or enterotyphlocolitis with necrotizing cryptitis was the most frequent lesion in calves infected with any of the serotypes, and tubulointerstitial nephritis and fibrinosuppurative splenitis were significantly associated with *S*. Dublin. We describe for the first time a high frequency of 17 virulence genes in bovine *S. enterica* Typhimurium and Dublin strains. Characterization of the virulence genes of *Salmonella* in cattle and their association with lesions is still scarce. The inclusion of a greater number of isolates could shed light on the virulence battery of *Salmonella*, which could eventually allow us to make associations with clinicopathological manifestations and epidemiological data.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s42770-022-00898-9.

Acknowledgements The authors thank A. Martínez and L. Betancor from the "Departamento de Desarrollo Biotecnológico, Instituto de Higiene, Facultad de Medicina, Universidad de la República" and Y. Perdomo and C. Monesiglio from INIA for collaboration in this work. We also thank the referring veterinarians and farmers.

Author contribution MLC, MF, and FG conceived the study. MLC, COS, RAC, MMR, RDC, CSS, VA, BDD, and FG performed the autopsy. MLC performed the bacteriological and molecular laboratory tests. COS, RAC, MMR, CSS, VA, BDD, and FG performed the microscopic analysis. WSN and FG analyzed data. MLC, MF, and FG wrote the first draft and final version of the manuscript. All authors read, edited, and approved the final version of the manuscript.

Funding This work was partially funded by INIA's grant PL-15, project FMV-104922 from the Uruguayan "Agencia Nacional de Investigación e Innovación" (ANII) and ANII (POS_NAC_2016_1_130296).

Data availability The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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