Leptospirosis as a cause of infertility in Uruguayan beef cattle

María V. Macchi[a,*], mmacchi@mgap.gub.uy, Alejandra Suanes[a], Ximena Salaberry[a], Bruno E. Dearmas[b], Emiliano Rivas[a], José Piaggio[b], Andrés D. Gil[b]

[a]División de laboratorios Veterinarios, Ministerio de Ganadería, Agricultura y Pesca, Montevideo, Uruguay

[b]Departamento de Bioestadística, Facultad de Veterinaria, Montevideo, Uruguay

*Corresponding author.

Abstract

Leptospirosis, caused by pathogenic spirochetes of the genus *Leptospira spp.*, is a globally significant zoonotic disease that affects humans and animals. In cattle, leptospirosis is associated not only with overt clinical manifestations but also with reproductive diseases, including infertility. This study assesses the potential correlation between leptospirosis and infertility in Uruguayan beef cattle. A case-control study involved 31 beef herds with no prior history of *Leptospira* vaccination. In each herd, veterinarians identified 10 non-pregnant (cases) and 25 pregnant cows (controls) using ultrasound, and blood and urine samples were collected from each cow. Serological diagnosis was performed using the Microscopic Agglutination Test (MAT), and quantitative PCR (qPCR) was used to assess *Leptospira* excretion. Additionally, antibodies against bovine viral diarrhea virus (BVDV) and infectious bovine rhinotracheitis (IBR) were tested. The results demonstrated an association between seropositivity to the Sejroe serogroup (cut-off 1:200) and infertility in cattle (OR=1.31; p-value=0.06). Furthermore, the level of *Leptospira* excretion (qPCR) in urine was associated with increased infertility risk, with cows excreting over 100 copies per mL of urine having the highest odds of infertility (OR=2.34; p-value<0.01). This study suggests a potential association between leptospirosis and infertility in Uruguayan beef cattle, emphasizing the importance of both serological and molecular diagnostics for assessing reproductive health in cattle herds. Future research should explore the impact of *Leptospira* serogroups on other reproductive disorders in cattle.

Keywords:

Bovine leptospirosis, Reproductive failure, Beef farms

1 Introduction

Leptospirosis, caused by pathogenic spirochetes of the genus *Leptospira spp.*, poses a widespread and significant global health threat. This bacterial pathogen, with its wide distribution, affects both humans and animals, highlighting its substantial importance as a zoonotic disease (Alder *et al.*, 2011). The disease's ubiquity across diverse climatic zones and geographical regions underscores its adaptability and the critical need for comprehensive understanding and management (Costa et al., 2015). Leptospirosis is renowned for its substantial impact on livestock, thus on agricultural industries, primarily due to its association with reproductive losses in various species (Boqvist et al., 2002; O'Doherty et al., 2015).

Amongst livestock, cattle are particularly susceptible to the detrimental effects of leptospirosis. The infection's repercussions extend beyond overt clinical manifestations. Reproductive losses in cattle are of great significance and have been extensively studied by numerous researchers (Adler and de la Peña Moctezuma, 2010). Although the occurrence of abortion, fetal death, premature births, and the birth of weak and/or underweight calves are recognized (Sanhueza et al., 2013; Grooms and Bolin, 2005); the study of chronic infections (primarily by serovar Hardjo) has revealed a notable incidence of infertility, repeated estrus and early embryonic death in affected animals (Loureiro & Lilenbaum, 2019; Junqueira et al., 2006). Such that approximately 8% of fetal losses was attributed to *Leptospira* (Sanhueza et al., 2013), as well as an observed increase of about 15% in estrus repetition in infected herds (Libonati et al., 2018).

In Latin America, infections caused by strains of Sejroe serogroup, particularly Hardjo serovar, have been frequently described in cattle (da Silva Pinto et al., 2016). Hardjo serovar is considered to be adapted to cattle, causing a silent and chronic disease that results in subclinical reproductive failure (Loureiro and Lilenbaum, 2020). In Uruguay, investigations have identified various circulating strains, including *L. interrogans* serogroup Pomona, *L. borgpetersenii* serogroup Sejroe, *L. noguchii* serogroup Autumnalis, *L. noguchii* serogroup Australis, *L. noguchii* serogroup Pyrogenes, *L. interrogans* serogroup Canicola, and two strains of *L. noguchii* that remain undetermined (Zarantonelli et al., 2018), with their reproductive impact unknown. This study has assessed the potential association between infertility and seropositivity to local strains and the urinary excretion levels of pathogenic leptospires in non-vaccinated beef cows.

2 Materials and methods

2.1 Ethics statement

Blood and urine samples were taken from the cattle following international recommendations for animal welfare, and with approval granted by the Ethics Committee for the Use of Animals for Experimentation (Comisión de Ética en el Uso de Animales de Experimentación, CEUA), DILAVE (División de Laboratorios Veterinarios), and the Ministry of Livestock, Agriculture and Fisheries (Ministerio de Ganadería, Agricultura y Pesca, MGAP, Uruguay).

2.2 Study design

A case-control study was designed to investigate the association between infertility in beef cattle and antibody titers against various local strains, alongside the urinary excretion levels of pathogenic leptospires. Eligible herds were selected in collaboration with 13 private practice veterinarians, who routinely refer samples to the laboratory and worked on herds that met the specified criteria. A total of 31 herds were selected using convenience sampling and based on the following characteristics: ensuring they had not been vaccinated against leptospirosis in the last five years, the use of ultrasonography for pregnancy diagnosis, and the upkeep of reliable information records. Breeding practices, whether natural or artificial, typically occurred over approximately three months, from December to February. All cattle are compulsory vaccinated every year against foot-and-mouth disease in February and May. These herds also had no history of vaccination for other reproductive diseases and predominantly consisted of Hereford or Angus cattle. Sampling of beef herds was conducted during the months of March and April from 2020 to 2022 all across Uruguay.

The sample size calculation was performed using STATA/IC 2015 software, employing the estimation of sample sizes for a two-sample proportions test. Assumptions included a prevalence range in the country of 25-50% thus a prevalence of 37% was used. The odds ratio (OR) for cases compared to controls was set at two, and a design effect (due to herd clustering) of three was assumed. These calculations were made with a power level of 0.8 and a precision of 0.95, resulting in 102 cases and 255 controls, respectively, which was considered the minimum sampling size.

Veterinarians performed ultrasound examinations to determine the pregnancy status of the cows within a two-month window after the service period concluded. Cows diagnosed as non-pregnant by ultrasound, indicating infertility, were identified as cases. The cows confirmed as pregnant by ultrasound, were defined as controls. In each herd, a randomized process led to the selection of 10 infertile cows and 25 pregnant cows. Blood and urine samples were collected from these animals on the same day of the ultrasound. A total of 303 infertile cows and 778 pregnant cows were sampled.

2.3 Sample management and testing

Blood samples were collected via venipuncture of the coccygeal vein in tubes without anticoagulant, and transported under refrigeration. The sera were then stored at 20 C. Prior to the collection of urine, each individual cow underwent intramuscular administration of diuretics (~150 mg furosemide, Furo R, Ripoll) and thorough genital organ cleansing

(wiping with 70% ethanol). Approximately 60 mL of midstream urine was collected in sterile 120 mL containers and transported at 4 C to the laboratory with the corresponding blood/serum samples.

The sera samples were employed to assess anti-*Leptospira* titers using the Microscopic Agglutination Test (MAT), following established protocols (Faine et al., 1999). Routine MAT tests used the national guide of positivity cut-off at titers 1:200. The panel of antigens used consisted of local strains from serogroups: Sejroe, Pomona, Autumnalis, Australis, Pyrogenes, and Canicola; in addition to two unidentified local strains of *noguchii* species (Zarantonelli et al., 2018). A sample was categorized as positive for this *L. noguchii* if it displayed reactivity to at least one of the strains. In samples where coagglutinations occurred between serogroups, the sample was considered seropositive for both serogroups if the difference between dilutions did not exceed one dilution. For those who exceeded one dilution, the serogroup with the highest dilution was considered seropositive.

Quantitative polymerase chain reaction (qPCR) amplification of lipL32 was performed using purified DNA extracted from 10 mL of the bovine urine samples, following the established protocols by Nieves Álvarez (2018). LipL32 PCR amplification was achieved using oligonucleotide primers (5′- lipL32–188 F TAAAGCCAGGACAAGCGCC-3′) and (5′-TACGAACTCCCATTTCAGCG-3′) lipL32–270R . The qPCR was performed in 10 µL FastSart Universal SYBER Green Master, 0.20 µg/µL bovine serum albumin (Sigma), 0.2 µL oligonucleotide primers (gBlock Gene Fragment - 1000 copies/µL), 5.8 µL H2O DNAsa/RNAsa Free and 2 µL template DNA. PCR cycling comprised of 1 denaturation step (10 min at 95 C), 40 amplification cycles (each cycle 30 s at 95 C and 30 s at 60 C), and a final cycle for the melting curve (15 s at 95 C, 15 s at 60 C, and 15 s at 95 C). The qPCR detection limit was 10 copies of Leptospires. Upon obtaining the quantification data, adjustments were made to normalize the number of leptospires to 1 mL of urine. These adjustments took into consideration the elution volume and the volume of DNA utilized in the PCR (100 µl), the volume of urine used for extraction (10 mL), reaction (2 µl).

Additionally, the assessment of antibodies against bovine viral diarrhea virus (BVDV) and infectious bovine rhinotracheitis (IBR) was conducted to be factored into the modeling process, given their reported associations with cattle infertility. For the detection of BVDV antibodies, a commercial competitive ELISA kit targeting (NSP2-3) p80–125 (anti-NSP2–3)antibodies (ID Vet, Grabels, France) was employed. The sample/negative ratio (S/N %) was calculated as

$$\frac{S}{N}\% = \left(\frac{OD_{sample}}{OD_{NC}}\right) \times 100$$

Where OD_{sample} was the optical density of the sample and OD_{NC} was the optical density of the negative control. Samples were considered positive when the S/N%≤ 45%. Regarding IBR, an indirect ELISA kit (ID Vet, Grabels, France) was utilized to detect anti-BHV-1 antibodies. The sample/positive ratio (S/P %) was calculated as

$$\frac{S}{P}\% = \left(\frac{OD_{sample} - OD_{NC}}{OD_{PC} - OD_{NC}}\right) \times 100$$

Where OD_{sample} was the optical density of the sample, OD_{NC} was the optical density of the negative control, and OD_{PC} was the optical density of the positive control. Samples were considered positive when the S/N% ≥ 55%. Both kits were executed in strict accordance with the protocols provided by the manufacturer.

Lastly, a comprehensive traceability system encompassing all cattle via electronic ear tags has been legislatively implemented in Uruguay. This system ensures the availability of detailed information for each cow. Thus, the age of each cow was documented.

2.4 Data analysis

A mixed effects logistic regression model was performed for multi-variable analyses using STATA/IC 2015 (StataCorp, 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC). The dependent variable was categorized as pregnant or infertile cows, while the variable 'herd' was treated as a stratum variable (random effect). All analyses were carried out at the individual cow level. For continuous independent variables, linearity with the outcome was assessed. In cases where discernible evidence of non-linearity was identified, the independent variables were appropriately categorized.

The strength of the association between the dependent variable and the independent variables was assessed through univariate analysis. The independent variables taken into account for the analysis were: seropositivity to each serogroup of *Leptospira* (cut-off 1:200), qPCR, seropositivity to IBR, seropositivity to BVDV, and the age of the cows. Covariates demonstrating p-values ≤ 0.2 in the chi-squared test were considered for potential inclusion in the multivariable model. Collinearity among variables was evaluated using Spearman's correlation coefficient. If two variables exhibited a correlation coefficient greater than 0.7, the variable most strongly linked to the outcome was retained for further analysis.

The final multivariate logistic regression model was manually constructed using the forward method. Variables were added to the model based on the significance of the p-values derived from the adjusted Wald test. The significance of the full model was ascertained using the Akaike Information Criterion (AIC). The interclass correlation coefficient (ICC) was calculated using the formula proposed by Dohoo et al. (2003), where σ_h^2 was the variance of the herd random effect:

$$ICC = \frac{\sigma_h^2}{\sigma_h^2 + \pi^{2/3}}$$

Furthermore, interaction terms between independent variables with biological plausibility were examined. For this purpose, Mantel-Haenszel odds ratios (OR) were calculated to perform the test of Homogeneity. Interactions were assessed in cases where the p-value was less than 0.05,. Potential confounding effects were investigated by monitoring alterations in the point estimates of the variables retained in the model. Changes in parameter estimates exceeding 20% were regarded as indicative of confounding and were kept in the model.

3 Results

Samples were collected from 303 infertile cows and 778 pregnant cows across 31 beef herds spanning ten of Uruguay's 19 departments. While one herd exclusively practiced artificial breeding, the remaining herds employed a combination of artificial and natural breeding methods.

The description of results for each variable and for each study group is detailed in Table 1. Regarding coagglutinations, they were observed in only 3.3% of the samples (36 samples), and these did not show differences in dilutions greater than one. Therefore, in these cases, they were considered positive for all coagglutinating serogroups.

alt-text: Table 1

Table 1

Univariate analysis of independent variables associated with infertility in beef cows in Uruguay.

Variables	Frequency %			*p-value*	OR (95% CI)
	Case cows	Control cows	Total		
Seropositivity to leptospirosis serogoups (cut-off 1:200)					
Sejroe	38.9	31.5	33.6	0.02	1.38 (1.05–1.82)
Pomona	4.6	4.0	4.2	0.47	1.16 (0.61–2,23)
Autumnalis	1.0	1.3	1.2	0.69	0.77 (0.21–2.81)
Australis	1.0	1.9	1.7	0.29	0.51 (0.15–1.77)
Pyrogenes	0	0	0	-	-
Canicola	1.7	1.4	1.5	0.77	1.17 (0.34–3.40)
Noguchii species (serogroup ND)	0.3	0.6	0.56	0.54	0.51 (0.06–4.40)
Copies of leptospires/mL of urine					
No copies detected (< 10)	61.1	71.9	68.8	-	*baseline*
10–100 copies	32.0	25.2	27.1	<0.01	1.49 (1.11–2.01)
> 100 copies	6.9	3.0	4.1	<0.01	2.76 (1.49–5.10)
Seropositivity to IBR	51.5	60.7	58.2	<0.01	0.68 (0.52–0.89)
Seropositivity to BVDV	69.0	72.4	71.4	0.27	0.85 (0.64–1.13)
Age of cows (month)					
< 36 month	19.5	20.4	20.2	-	*baseline*
36–60 months	61.1	57.7	58.6	0.55	1.11 (0.79–1.57)
> 60 month	19.5	21.9	21.2	0.75	0.94 (0.61–1.42)

OR (95% CI): odds ratio and 95% confidence interval. ND:

alt-text: Table 2

Multivariate analysis of potential risk factors associated with beef cows infertility in Uruguay.

Variables	OR	95% CI	*p-value*

Seropositivity to Sejroe serogroup (≥1:200)	1.31	0.99–1.75	0.06
Copies of leptospires/mL of urine			
No copies detected (< 10)	-	-	-
10–100 copies	1.40	1.04–1.90	0.03
> 100 copies	2.34	1.25–4.39	< 0.01
Seropositivity to IBR	0.70	0.53–0.92	0.01
Intercept	0.38	0.30–0.48	< 0.01

95% CI: 95% confidence interval.

In the univariate analysis, seropositivity to the Sejroe serogroup (1:200), copies of leptospires per mL of urine (qPCR), and seropositivity to IBR exhibited a statistically significant associations with infertile cows (Table 1). Although seropositivity to BVDV and the age of the cows yielded a p-value > 0.2, it was still included in the multivariate analysis due to its potential role as a confounding variable. Continuous variables had no evidence of a linear relationship with the outcome, so they were categorized as shown in Table 1. No correlation was found between the independent variables.

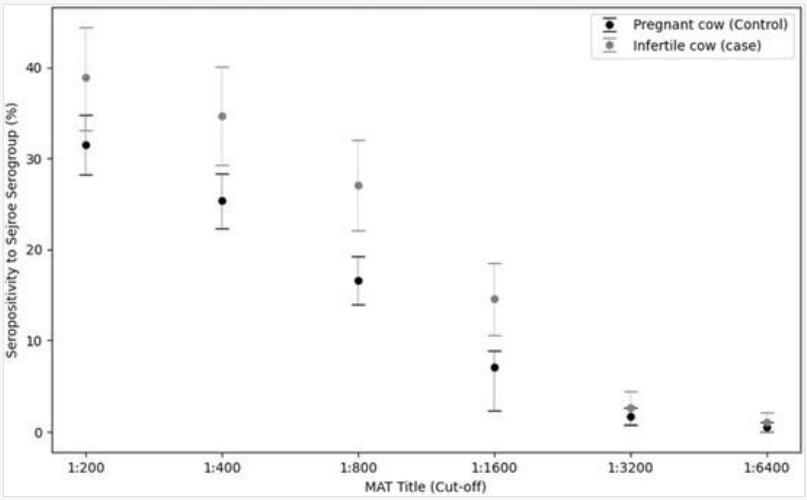
In the final model, employing mixed-effect logistic regression with herds as a random effect, three variables were incorporated (Table 2). In addition, the estimated variance of the herd random effect was 9.84e-35, and the ICC was 2.99e-35.

Infertile cows had 1.31 times the odds of being seropositive to Sejroe serogroup (cut-off 1:200) than pregnant cows. A significant association was found with qPCR, the risk of cows being infertile increased with the levels of pathogenic leptospires excreted in urine. Cows excreting ≤100 and >100 copies of leptospires per mL of urine had 1.40 and 2.34 times higher odds of infertility, respectively, than cows with no detectable copies of leptospires in their urine. IBR antibodies were negatively associated with infertility; nevertheless, keeping it in the model was necessary due to its potential impact on the outcome. No interaction or confounding effects were observed.

In a descriptive analysis, different cut-off values for MAT were evaluated for the Sejroe serogroup (Fig. 1). As the cutoff values increased, the differences between pregnant and infertile cows became more pronounced, with noticeable disparities observed up to the cut-off of 1:1600. However, beyond this point, these differences are no longer evident, although it is important to note that the seropositive levels at this cut-off were quite low.

Fig. 1

Fig.



Percentage of seropositive results based on different cut-off values in the MAT for the Sejroe serogroup, distinguishing between infertile cows (cases) and pregnant cows (controls). The figure displays the mean and a 95% confidence interval.

4 Discussion

In recent years, leptospirosis has emerged as a significant concern in the context of reproductive health in cattle. Research has highlighted its potential role in causing embryonic death and subfertility (Lilenbaum and Martins, 2014). Notably, *Leptospira* bacteria have been identified in renal tissues and within the female reproductive tract, giving rise to a silent yet chronic reproductive disease (Loureiro and Lilenbaum, 2020).

This study conclusively established the association between leptospirosis and infertility in beef cattle. In Uruguay, leptospirosis has become endemic, with seroprevalence rates ranging from 25% to 50% at the individual cow level and 50–87% at the herd level (Caffarena et al., 1971; Repiso et al., 2005; Suanes et al., 2024). Another study conducted on 48 beef and dairy herds confirmed the widespread presence of the bacterium in the country, with a herd-level excretion rate of 20%. Cows with a history of abortions were sampled, resulting in the isolation of 40 strains, including *L. interrogans* serogroup Pomona serovar Kennewicki, *L. interrogans* serogroup Canicola serovar Canicola, *L. borgpetersenii* serogroup Sejroe serovar Hardjo, and nine strains of *L. noguchii* (Zarantonelli et al., 2018). While this last study confirmed the circulation of *Leptospira* among herds with a reproductive history of abortions, its impact on unvaccinated farms and asymptomatic cows was unknown.

The serogroup Sejroe was associated with infertility in cattle. Evaluation of cut-off values revealed an association from a titer of 1:200, which grew stronger as titers increased, peaking at 1:1600. This suggests that recent infections may pose a higher risk of infertility in cows. Even though it is well-documented that chronically infected animals with serogroup Sejroe typically show low MAT titers (<1:100) (Orjuela et al., 2022), antibody titers may still be indicative of infertility rates in cattle. Numerous studies have reported associations between the Sejroe serogroup and reproductive problems, such as infertility, early embryonic death (Grooms and Bolin, 2005), and estrus repetition (Libonati et al., 2018). In a study conducted in Brazil, herds with >20% prevalence of anti-Sejroe antibodies exhibited 39.9% more embryonic deaths than those with low prevalence; the embryonic death diagnoses were confirmed by ultrasonography to ensure the accuracy of this measure (Oliveira et al., 2021).

The low prevalence of reactive animals to the other local strains might suggest that bovines are minimally affected by these infections, undergoing asymptomatic courses despite the previously described circulation of these strains in the country (Suanes et al., 2024). In recent years, *L. noguchii* isolates have been reported, mainly in asymptomatic cattle (Zarantonelli et al., 2018; Martins et al., 2015). Although abortions caused by *Leptospira noguchii* have been observed in small ruminants, there is currently no evidence of this species inducing infertility in cattle (Aymée et al., 2022).

Levels of pathogenic *Leptospira* excretion were associated with infertility in beef cows. Cows with higher excretion levels had a greater risk of experiencing infertility. It is important to note that, in a previous study, a 20% excretion rate was reported in cattle (Zarantonelli et al., 2018). However, it is worth highlighting that the PCR technique used in this study had a lower detection limit (10 *Leptospira* copies *vs*. 100 copies) due to qPCR. When considering only the percentage of cows with excretion levels exceeding 100 *Leptospira* copies, it was significantly lower in this study (4.1% *vs*. 20%). This disparity may be attributed to the fact that the study by Zarantonelli et al. (2018) selected farms with a history of abortions, so the selected herd would have a higher probability of *Leptospira* excretion and dissemination within the herd.

Although both serological diagnosis and urine *Leptospira* excretion were successfully integrated into the multivariate model, no interaction was found between these variables. This absence of interaction might be due to the intermittent pattern of urine excretion, as previous research has shown no correlation between positive leptospirosis serology and the detection of the pathogen in urine (Otaka et al., 2012). A study across multiple domestic animal species in Brazil, involving 512 animals, revealed no apparent correlation between positive PCR results in urine samples and antibody titers in the MAT. These findings indicated that seroreactivity was not significantly associated with PCR results, when considering overall seroreactivity and when sera were categorized by titers (Hammond et al., 2014). Additionally, it is noteworthy that cattle may not produce a reaction against their own isolates (Libonati et al., 2017),highlighting the lack of correlation between serology and excretion.

The development of the model took into account viral infections, widely recognized as causes of infertility in cattle (Givens, 2006). It is essential to acknowledge that reproductive failure is a multifactorial condition.

Despite the relatively low odds ratio (OR<1) for IBR, which is a recognized cause of infertility in cattle due to its high seroprevalence worldwide (Mare et al., 1961; Mahmoud et al., 2009; Nettleton and Russell, 2017), this study found no clear explanation for the higher risk of infertility in seronegative cows at the commercial cut-off. The persistence of antibodies against viral infections, which can protect the cattle population from new infections, is well-documented in seropositive cows. While the endemic nature of IBR was evident in 58.2% of seropositive cattle, this does not entirely account for the infertility risk among seronegative cows. Considering the potential immunocompromised status of some seronegative cows and the need to validate cut-off points in regions with high viral circulation, further research is warranted to elucidate these associations.

Considering the study's design, it's noteworthy that the classification of infertility occurred concurrently with pregnancy diagnosis, potentially introducing a misclassification bias. This potential bias stems from the prospect of early embryonic death, for which data collection was not feasible. However, leptospirosis in cattle, caused by adapted strains, is more commonly associated with milder conditions, including subfertility and early embryonic death (Loureiro & Lilenbaum, 2019). In this study, cases encompassed both types of reproductive disorders. Additionally, due to the study design, it's not possible to determine whether leptospiral infection occurred before or after infertility onset.

Another potential source of bias is the omission of body condition score (BCS). Previous research has linked BCS to increased early pregnancy loss (Zobel et al., 2011) and mid-late abortions (Sanhueza et al., 2013). Neglecting this variable may have impacted the study's outcomes, emphasizing the importance of its inclusion in future research to gain a more comprehensive understanding of the factors contributing to reproductive losses in cattle.

The study underscores the significant public health implications of leptospirosis, especially in countries with strong livestock industries like Uruguay. High levels of leptospiral excretion in urine highlight the urgent need for increased awareness among rural workers and those involved in livestock production. This emphasizes the occupational risk and the importance of targeted preventive measures such as education, vaccination campaigns and improved hygiene practices in agricultural settings to mitigate transmission and protect human and animal health (Schelotto et al., 2012).

5 Conclusion

The study’s findings revealed a heightened risk of infertility in seropositive cows and those excreting leptospires in their urine. The combined use of serological and qPCR diagnoses is recommended as valuable tools for assessing infertility in beef herds. Given these findings, it is crucial to emphasize the importance of vaccination as a preventive measure. Although no clear association was identified with other Leptospira serogroups, further research should explore their potential impact on other reproductive diseases, such as abortions and the birth of weak or stillborn calves. These strains are known to be non-adapted to cattle, and these conditions are more strongly associated with this type of infection.

CRediT authorship contribution statement

Ximena Salaberry: Investigation. **Alejandra Suanes:** Writing – review & editing, Project administration, Methodology, Investigation. **María Valentina Macchi:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Andrés Domingo Gil:** Supervision, Methodology, Investigation. **José Piaggio:** Methodology, Investigation. **Emiliano Rivas:** Investigation. **Bruno Esteban Dearmas:** Investigation.

Declaration of Competing Interest

The authors declare no conflict of interest with the outcomes of this study.

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