Epidemiology Aspects of *Brucella*-DNA Detection Using Real-time PCR from Bovine Whole Blood Samples in Colombia

Olga Lucia Herrán Ramírez
Federal Rural University of Rio de Janeiro: Universidade Federal Rural do Rio de Janeiro
https://orcid.org/0000-0002-4783-0269
Huarrisson Azevedo Santos
Federal Rural University of Rio de Janeiro: Universidade Federal Rural do Rio de Janeiro
Patrícia Gonzaga Paulino
Federal Rural University of Rio de Janeiro: Universidade Federal Rural do Rio de Janeiro
Carolina Soares van der Meer
Federal Rural University of Rio de Janeiro: Universidade Federal Rural do Rio de Janeiro
José Luis Rodríguez Bautista
Federal Rural University of Rio de Janeiro: Universidade Federal Rural do Rio de Janeiro
Ingrid Lorena Jaramillo Delgado
CES University: Universidad CES
Juliana Gonzales Obando
University of Antioquia: Universidad de Antioquia
Rene Ramirez Garcia
CES University: Universidad CES
Isabele Da Costa Angelo (isabeleangelo@yahoo.com.br)
Federal Rural University of Rio de Janeiro: Universidade Federal Rural do Rio de Janeiro
https://orcid.org/0000-0003-3698-8340

Research Article

Keywords: Bacterial zoonoses, brucellosis, diagnosis, whole blood

Posted Date: April 26th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-441208/v1

Abstract

A cross-sectional study was conducted in Colombia to recover *Brucella* spp. DNA from bovine whole-blood samples through probe-based real-time PCR (Probe-qPCR) and by an SNP-based assay, differentiating vaccine strains from field strains. The associated factors for the presence of *Brucella*-DNA were reported and evaluated using logistical regression models. A total of 656 random cows from 40 herds were selected. Template DNA was obtained based on a modified salting-out protocol. The Probe-qPCR assay using *bcsp*31 gene amplification showed an efficiency of 92.35%, with a slope of -3.52 reached in the standard curve. The qPCR assay detected 9.5% (n = 62/656; 95% CI: 7.3,12.0) of the animals with *Brucella*-DNA presence, and 62.5% (n = 25/40; 95% CI: 45.8,77.3) of the herds with *Brucella*-DNA presence. Using the SNP-based assay, all positive samples were identified as field *Brucella* strains. In the final regression model at the animal-level, five variables were associated with *Brucella*-DNA presence: the use of bulls for mating, recorded history of reproductive problems, pregnant cows, parlor milking, and cows belonging to farms ≤ 200 m from the main road. At the herd-level, two variables were associated with *Brucella*-DNA presence: recorded history of reproductive problems and bulls' use for mating. Given the fluctuant brucellosis prevalence in endemic areas, updated epidemiological studies are necessary to evaluate the disease dynamic, and if established prevention and control measures have been effective or need to be adjusted. The increase in the prevalence of brucellosis in animal reservoirs creates an important risk of transmission in humans.

Introduction

Brucella spp. are the causative agents of brucellosis, a complex and worldwide zoonotic disease that has been neglected for a long time. According to the Plan of Action for eliminating Neglected Infectious Diseases (NIDs) 2016–2022, there continues to be a need to evaluate and document the regional epidemiological situation concerning brucellosis for the Americas. Although presenting endemic brucellosis, Latin America reports insufficient data on the prevalence and has not yet reached satisfactory levels of disease control (World Health Organization and Pan American Health Organization, 2016). Depending on the prevalence found, the strategies and control measures could apply different management actions (Moreno, 2020). Furthermore, proper detection can help mitigate the economic impact generated by the culling of infected animals (Trotta et al., 2020), the costs to recover a brucellosis-free status, the socio-economic impact on rural development, and more importantly, the public health impact.

Bacterial culture is considered the “gold standard” to confirm brucellosis' diagnosis. Nevertheless, most *Brucella* species are fastidious and relatively slow-growing organisms, affecting in some cases, the detection sensitivity (World Organization for Animal Health, 2018). Instead, epidemiological studies targeting brucellosis in livestock are based almost exclusively on serology tests as the main detection tools due to their rapid results and low cost. However, serological tests present low sensitivity in early or latent stages of infection and lack specificity in regions where brucellosis is highly endemic, other bacteria share the structure of O-LPS, or there is massive cattle vaccination (Godfroid et al., 2002; Corbel,

2006). In countries with massive vaccination, the reliable differentiation between vaccine strains and field strains of *Brucella* sp. is crucial for epidemiological studies of bovine brucellosis. (Gopaul et al., 2010). Therefore, better tests and testing strategies, adapted to the epidemiological situation, need to be developed and validated (Godfroid et al., 2002).

Improvements in PCR methods such as real-time PCR (qPCR), have made it possible to detect *Brucella* spp. from clinical samples of human (Queipo-Ortuño et al., 1997; Baoshan et al., 2020), and animal (Wareth et al., 2015; Gwida et al., 2016; Selim et al., 2019), even when there is a low number of organisms (Hull et al., 2018), combining speed, sensitivity, high specificity (with use of probe), laboratory safety, and low risk of cross-contamination or amplicon contamination in the bloodstream (Corbel, 2006). The qPCR technique's application may support the drawbacks of the indirect methods and the low sensitivity of the microbial isolation technique.

While there is not significant available data on the prevalence of brucellosis in humans, the increase in the prevalence of brucellosis in animal reservoirs creates an important risk of transmission in humans. The Control and Prevention Program of Bovine Brucellosis in Colombia has been conducted based on the detection of positive animals by indirect methods, combined with eradication and mandatory vaccination strategies (Instituto Colombiano Agropecuario, 2017).

This cross-sectional study evidences the associated bovine brucellosis factors from Brucella DNA from bovine whole blood, differentiating vaccine strains from field strains by an SNP-based assay.

Materials And Methods

2.1. Design and area of study

A cross-sectional study on bovine brucellosis was conducted in four municipalities of Antioquia, Colombia: Santa Rosa de Osos (6°38'50"N 75°27'38"O), Entrerrios (6°33'55"N 75°30'51"O), Belmira (6°36'18"N 75°39'57"O), and San Pedro de Los Milagros (6°27'34"N 75°33'28"O). This geographical area is Colombia’s leading bovine milk production region, and mechanical milking systems are predominant. The altitude of the study area ranged from 2,400 to 2,850 meters above sea level. Specialized dairy breeds such as Holstein, Jersey, Brown Swiss, Ayrshire, and specialized breed crosses, are used in the selected area. Milk production mean is 18.4 litres/cow in two milkings. Most farms use parlors as milking sites (Bedoya et al., 2018).

2.2. Sampling and target population

The sampling procedure applied the formula $n = 1.962\ p\ (1 - p)/d2$ to calculate the sample size with a confidence level of 95%, desired absolute precision (*d2*) of 5%, and expected prevalence (*p*) of 10% (Thrusfield, 2007). A total of 656 whole-blood samples were randomly collected from cows in 40 dairy herds of four municipalities in the north of Antioquia. Proportionate sampling was used from each city to ensure the number of dairy cattle has not been underrepresented.

The target population was composed of animals in lactating or in the dry period, ≥ 2-year-old, vaccinated at the age of 3 to 8 months, mainly with 10–34 x 10^9 colony forming units (CFU) of rough attenuated strain RB51 or alternatively with 20 x 10^9 CFU of smooth live vaccine strain S19. Mandatory vaccination was verified by official vaccination records (RUV) (Instituto Colombiano Agropecuario, 2017). The animal's data was taken from the Foot and Mouth Disease and Brucellosis vaccination campaign (Instituto Colombiano Agropecuario, 2018a). The herds' selection was made considering the producers' availability to participate voluntarily in the research and signed the informed consent agreement. Each farmer gave a full animal list to select the participants. A random number table without replacement was used in the cow's election. From herds with ≤ 50 cows, 15 dairy cows were chosen for the current research. From herds with > 50 cows, 22 dairy cows were selected. The randomly chosen animals were transferred to a designated handling area within each farm for collecting the samples. The animal skin area was cleaned with chlorhexidine and six milliliters of caudal venous blood were taken from each animal using blood collection tubes with ethylenediaminetetraacetic acid, an anticoagulant. The blood samples were refrigerated and properly transported to the lab.

2.3 Epidemiological data collection

Dairy farmers were asked to answer a questionnaire. The questionnaire was based on a literature review and included closed questions regarding cattle management, zootechnical parameters, and sanitary practices as independent variables (Tables 1 and 2). The questionnaire was pre-tested and validated by farm managers that did not belong to the study.

Table 1
Descriptive and Univariable analysis at the animal level between independents variables and *Brucella* spp. infection of 656 cows in 40 herds located in dairy cattle of Antioquia, Colombia.

	Descriptive analysis		***Univariate analysis***		
Variable	**Frequency**	**%**	x^2	p**	**OR (IC 95%)**
Age			1.112	0.573	
24–36 months	11/150	17.7			
37–60 months	25/258	40.3			
> 60 month	26/248	41.9			
Parity			0.847	0.655	
Primiparous	12/140	19.4			
Pluriparous	47/449	75.8			
Heifers	3/20	4.8			
Days of gestation			7.176	0.028	
0	18/257	29.0			
1–90 days	22/147	35.5			
> 90 days	22/252	35.5			
Pregnacy status			2.957	0.085	1.64 (0.93, 2.91)
Pregnant	44/399	71.0			
Not pregnant	18/257	29.0			
Lactaction cycle			0.580	0.810	0.916 (0.45, 1.87)
Lactanting period	52/557	83.9			
Dry period	10/99	16.1			
Municipalities			23.579	0.000	
Belmira	6/93	9.7			
Entrerrios	15/169	24.2			
San Pedro de los Milagros	23/105	37.1			
Santa Rosa de Osos	18/289	29.0			

%: percent of positive; *p-value*: statistical significance; OR: odds ratio; (95% CI): confidence interval with $p^{**} < 0.05$.

	Descriptive analysis		*Univariate analysis*		
Brucellosis status			10.726	0.005	
Positive status	25/157	40.3			
Brucellosis free status	17/264	27.4			
Unknown Status	20/235	32.3			
Type of vaccine strain			4.708	0.195	
Both strain	3/60	4.8			
Strain RB51	39/453	62.9			
Strain S19	10/99	16.1			
None	10/59	16.1			
Raising of calves at the farm			5.009	0.025	2.34 (1.09, 5.03)
No	54/495	87.1			
Yes	8/161	12.9			
Origin of sampled cow			1.213	0.545	
Purchased	22/196	35.5			
Transferred	7/93	11.3			
Born	33/367	53.2			
Recorded history of reproductive problems			8.227	0.004	4.06 (1.45, 11.39)
Yes	58/522	93.5			
No	4/134	6.5			
Abortion			0.271	0.603	1.25 (0.54, 2.87)
Yes	7/62	11.3			
No	55/594	88.7			
Birth of weak calves			1.320	0.242	2.44 (0.51, 11.761)
Yes	2/10	3.2		*F*	
No	60/646	96.8			

%: percent of positive; *p-value*: statistical significance; OR: odds ratio; (95% CI): confidence interval with $p^{**} < 0.05$.

	Descriptive analysis		*Univariate analysis*		
Retention of fetal membranes			0.018	1.00	0.87 (0.11, 6.84)
Yes	1/12	1.6		*F*	
No	61/644	98.4			
Maternity pen			0.614	0.433	1.24 (0.72, 2.13)
No	39/382	62.9			
Yes	23/274	37.1			
Mating type			13.300	0.001	
Both	31/207	50.0			
AI	8/186	12.9			
Bull	23/263	37.1			
Disposal of discarded milk			0.365	0.833	
Pen	22/255	35.5			
Septic tank	10/105	16.1			
Feed calf	30/296	48.4			
Milking process			10.061	0.007	
Milking in pen	7/67	11.3			
Mechanic milking system in pen	12/247	19.4			
Parlor Milking	43/342	69.4			
Co-grazing with another species			0.115	0.994	
Horses	46/481	74.2			
Small ruminants	3/38	4.80			
None	12/137	21.0			
Sharing of water sources			2.173	0.14	1.51 (0.87, 2.62)
Yes	41/376	66.1			
No	21/280	33.9			
Herds size			5.575	0.018	2.11 (1.12, 3.96)

%: percent of positive; *p-value*: statistical significance; OR: odds ratio; (95% CI): confidence interval with $p^{**} < 0.05$.

	Descriptive analysis		*Univariate analysis*		
> 50 animals	52/439	80			
≤ 50 animals	13/217	20			
Grazing density			7.370	0.007	2.076 (1.21, 3.55)
≥ 3.5 AU	38/295	61.3			
< 3.5 AU	24/361	38.7			
Main roads			14.216	0.000	2.69 (1.58, 4.57)
≤ 200 m	31/191	50.0			
> 200 m	31/464	50.0			

%: percent of positive; *p-value*: statistical significance; OR: odds ratio; (95% CI): confidence interval with $p^{**} < 0.05$.

Table 2
Univariable analysis at the herd level between independents variables and *Brucella* spp. infection of 656 cows in 40 herds located in dairy cattle of Antioquia, Colombia.

	Descriptive analysis		**Univariate analysis**		
Variable	**Frequency**	**%**	x^2	p**	**OR (IC 95%)**
Municipalities			5.867	0.118	
Belmira	3/6	12.0			
Entrerrios	6/9	24.0			
San Pedro de los Milagros	7/7	28.0			
Santa Rosa de Osos	9/18	36.0			
Brucellosis status			0.444	0.801	
Brucellosis free status	9/16	36.0			
Unknown Status	10/15	40.0			
Positive Status	6/9	24.0			
Type of vaccine strain			2.336	0.506	
Both strains	2/4	8.0			
Strain RB51	18/29	72.0			
Strain S19	2/4	8.0			
None	3/3	12.0			
Raising of calves at the farm			1.881	0.273	2.67 (0.64, 11.07)
No	20/29	80.0		*F*	
Yes	5/11	20.0			
Origin of cattle			1.881	0.273	2.67 (0.642, 11.07)
Purchased or transferred	20/29	80.0		*F*	
Born	5/11	20.0			
Recorded history of reproductive problems			6.000	0.036	7.67 (1.30, 45.29)
Yes	23/32	92.0			

%: percent of positive; *p*-value: statistical significance; OR: odds ratio; (95% CI): confidence interval with $p^{**} < 0.05$.

	Descriptive analysis		Univariate analysis		
No	2/8	8.00			
Abortion			0.104	1	1.31 (0.25, 6.88)
Yes	21/33	84.0		*F*	
No	4/7	16.0			
Birth of weak calves			4.302	0.060	7.87 (0.88, 70.15)
Yes	9/10	36.0		*F*	
No	16/30	64.0			
Retention of fetal membranes			0.064	0.800	1.18 (0.32, 4.42)
Yes	16/25	64.0			
No	9/15	36.0			
Maternity pen			0.064	0.800	1.18 (0.32, 4.42)
No	16/25	64.0			
Yes	9/15	36.0			
Mating type			4.397	0.111	
Both	10/12	40.0			
AI	4/10	16.0			
Bull	11/18	44.0			
Disposal of discarded milk			1.918	0.383	
Pen	9/15	36.0			
Septic tank	3/7	12.0			
Calf	13/18	52.0			
Milking process			0.924	0.630	
Milking in pen	4/5	16.0			
Mechanic milking system in pen	9/16	36.0			
Parlor Milking	12/19	48.0			

%: percent of positive; *p*-value: statistical significance; OR: odds ratio; (95% CI): confidence interval with $p^{**} < 0.05$.

	Descriptive analysis		Univariate analysis		
Co-grazing with another species			0.000	1	1.00 (0.20, 3.95)
Yes	20/32	80.0			
No	5/8	20.0			
Sharing of water sources			0.000	1	1.00 (0.27, 3.69)
Yes	15/24	60.0			
No	10/16	40.0			
Herds size			0.064	1	0.84 (0.23, 3.15)
> 50 animals	9/15	36.0			
≤ 50 animals	16/25	64.0			
Grazing density			2.462	0.117	2.98 (0.74, 11.93)
≥ 3.5 AU	13/17	52.0			
< 3.5 AU	12/23	12.0			
Main roads			10.286	0.001	2.15 (1.45, 3.20)
≤ 200 m	12/12	48.0		*F*	
> 200 m	13/28	52.0			

%: percent of positive; *p*-value: statistical significance; OR: odds ratio; (95% CI): confidence interval with $p^{**} < 0.05$.

2.4. DNA extraction from whole blood samples and quality analysis

Template DNA was obtained based on the non-organic salting out protocol designed for the recovery of DNA from whole blood samples described previously (Miller et al., 1988), with the improvement made by Queipo-Ortuño et al. (1997), and modifications by the authors (detailed descriptions of the extraction protocol are given as follows for reproducibility in supplementary material). The extracted DNA was preserved at − 20°C until the next evaluations. The template DNA's concentration and purity were assessed through a Nanodrop ND-2000® spectrophotometer (Thermo Fisher Scientific, USA). The ratio of absorbances A260 / 280 and A260 / 230 was used to evaluate the samples' purity. The integrity was assessed in a 0,8 % agarose gel. The gel was run at 75 volts for about 45 min and subsequently stained with ethidium bromide (0.5 mg/mL).

2.5. Molecular detection of Brucella spp.

Each DNA sample was analyzed in duplicate against positive and negative matrix controls. DNA of *B. abortus* S19 vaccine (Vecol S.A, Colombia) strain was used as positives standard control in all molecular assays and comprised 10^2 copies of *bcsp*31 gene. Negative controls were also included and contained all the elements of the reaction mixture, as well as molecular biology grade DNase and RNase Free water (RPI®) instead of DNA template. The TaqMan™ exogenous internal positive control (IPC) (Thermo Fisher Scientific, USA), was used to identify the samples with presence of inhibitors.

A specific sequence for *the Brucella* genus was chosen from the conserved region of the gene *bcsp*31, which encodes the 31-kDa immunogenic membrane protein BCSP31(GenBank: M20404.1) (Bricker et al., 1988). Baily et al. (1992) designed the primers: *bcsp*31-Forward 5'-TGGCTCGGTTGCCAATATCAA-3'; and *bcsp*31-Reverse 5'-CGCGCTTGCCTTTCAGGTCTG-3' were chosen for the qPCR assays. These primers amplify a 224 bp of the *bcsp*31 gene. Furthermore, the hydrolysis probe 5'-CCGGTGCCGTTATAGGCCCAATAGG-3', with dual labelled fluorescence at the 5'-end with 6-carboxyfluorescein phosphoramidite (FAM) as the reporter dye, and at the 3'-end with 5-carboxytetramethylrhodamine (TAMRA) as quencher was chosen to improve specificity (Sohn et al., 2003).

Optimum primer concentrations were determined by testing different primer concentration combinations with a qPCR in the presence of SYBR Green to obtain the maximum sensitivity. A melting curve analysis was performed after the amplification protocol on the green channel from 60–95°C with an increasing 0.3°C / s. The melting curve-generated peak represented the specific amplified product. A unique dissociation curve was produced to confirm the specificity of the amplification. Optimization of the probe concentration was conducted using several probe concentrations from 0.2 µM to 0.4 µM in qPCR assays with the optimized concentration of primers and a low level of the target (10^2 copies of *Brucella abortus*).

A qPCR assay was conducted to verify the optimum DNA amounts to be applied in the molecular survey. The DNA amount ranged from 1 µl to 3 µl and a DNA concentration between 125–375 ng. The template DNA volume optimum to add to the reaction mixture without creating the amplification inhibition was 1 µl. The optimum annealing temperature in the qPCR was 62°C. The IPC no revealed potential inhibitors in the template of DNA amplification.

The qPCR reactions were conducted at the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Thermo Scientific, USA) in a total volume of 12 µl, comprising of 6.0 µl of 1x Mix QuantiNova™ Probe PCR Master and Rox (Qiagen®, Germany), 0.6 µl of Mix 0.4 µM of each Primer, and 0.2 µM of TaqMan probe, 0.24 µl of 1X of Exo IPC Mix, 0.6 µl of 1X of Exo IPC DNA and approximately 125 ng of total DNA. The thermocycling conditions were performed as follows: one hold at 95°C for 2 min, followed by 45 cycles composed of denaturation at 95°C for 5 s, and annealing/extension at 62°C for 10 s. The samples with cycle quantification (Cq) values less than 40 cycles were considered positive. All laboratory biosafety and

one-way flow processes were applied to prevent contamination (Richardson et al., 2009). Blind sample processing was used, assigning a code to each sample, to avoid potential observer bias.

2.6. qPCR standard curve

The concentration of DNA extracted from the *B. abortus* strain present in the S19 vaccine (Vecol S.A, Colombia) was used to calculate the number of genome copies, assuming a genome size of 3.28 x 106 bps. The standard curve was built using six serial decimal dilutions of the purified template DNA (from 10^5 to 0.1 genome) to verify the reaction's limit of detection, precision, and efficiency. The copy number determination of the first dilution point followed the equation: Number of copies = (6.02 x 1023 (copies per mole) x DNA concentration (g)) / (*B. abortus* genome size (base pairs) x 660 (g / mol / bp)). The qPCR Efficiency was determined following the equation: E = 10(−1/slope)-1.

2.7. Differentiation of Brucella field strains from RB51 vaccine strains.

The qPCR assay previously designed by Gopaul et al., 2010 was used to distinguish the field strains from RB51 vaccine strain through a single nucleotide polymorphism (SNP) in the *Brucella* spp. The assay was set up in a reaction mixture volume of 12 µl comprising 1X of TaqMan™ Universal PCR Master Mix (Applied Biosystems, Delaware, USA), 0.8 µM of each Primer, and 0.3 µM of TaqMan probe and approximately 125 ng of total DNA. Reactions were run on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Thermo Scientific, USA) using thermocycling conditions of one hold at 95°C for 10 min followed by 50 cycles composed of denaturation 92°C for 15 s and annealing/extension 60°C for 1 min.

2.8. Spatial analysis

The information collected was recorded in an Excel file and transformed into a shapefile format to be worked in a geoprocessing tool as QGIS software (QGIS v 3.4.10 -Madeira) under the GNU General Public License and the European Petroleum Survey Group (EPSG) code 31978. The data was plotted on the cartographic database obtained from the National Department of Statistics Administration (DANE) from Colombia as SHP format. A distance of ≤ 200 m, > 200 m from these roads was used to identify the number of dairy farms in the area. Plotting of the dairy farms that were positive in the qPCR and the cELISA tests was performed using the Intersec tool. Positive farms were plotted using symbols of different size, proportional to the number of positive cases in each dairy farm, to identify areas with a higher number of points for each detection method.

2.9. Statistical analysis

The epidemiological data was analyzed using the statistical program IBM SPSS (Statistical Package for the Social Sciences) v. 25.0 for Windows (Armonk, Nueva York: IBM Corp.). Pearson correlation coefficient was used for quantitative variables with the normality assumption. Spearman's rank-order correlation

was applied when quantitative variables did not meet the normality assumption. A chi-square test or Fisher test was used to evaluate the association between *Brucella*-DNA presence by qPCR assay with all the explanatory variables. Statistically significant variables and variables with *p*-value < 0.25 were offered to the logistic regression models from individual animal and the herd level. Before creating the final model, the multicollinearity was measured by determining the variance inflation factors (VIF) and tolerance. Collinearity was considered when the VIF value exceeds 6.0, and the tolerance was less than 0.2. According to the literature review, when two variables were found with high collinearity, only the variable with more biological importance and plausibility was included in the model. The model was built by the "intro" method. Finally, the coefficients were estimated, and the appropriateness adequacy, validity, and usefulness of the model were identified.

Results

Descriptive analysis exhibited that the mean age of evaluated cows was 59.2 ± 24.6 months old. The mean gestational age was 81.3 ± 84.5 days, and the offspring's mean was 3.0 ± 1.9. The 84.9 % (n = 557) of the tested cows were in the lactation cycle, and 15.1 % (n = 99) in the dry cycle.

The qPCR's mean Cq value for the positive samples was 35.54 ± 2.1 cycles. The analytical sensitivity analysis showed a determination coefficient (R^2) equal to 0.99, and the Cq values ranged from 13.68 cycles in the first dilution point (10^5) to 34.66 cycles in the last dilution point (0.1 genome). An efficiency of 92.35% with a slope of -3.52 was reached in the standard curve (Fig. 1).

The *Brucella*-DNA prevalence at the animal level was 9.5 % (n = 62/656; 95 % CI: 7.3, 12.0). At the herd level, the *Brucella*-DNA prevalence was 62.5 % (n = 25/40; 95 % CI: 45.8, 77.3) (Fig. 2). The SNP-based assay confirmed that all positive samples were from *Brucella* spp. field strains.

The quantitative variables did not meet the normality assumption and were not statistically significant by Spearman's rank-order correlation. Therefore, they were transformed into ordinal variables in the model (Tables 1 and 2).

The bivariate analysis at the animal level showed that the variables: days of gestation, reproductive problems records, use of a bull or both (AI, bull) for the mating, parlor milking, mechanical milking in pen, herd size, grazing density, brucellosis status, municipality, and < 200 m of distance to the main road, were statistically significant variables with *Brucella*-DNA presence (*p*-value < 0.05). At the herd level, reproductive problems records, a bull for mating, and farms < 200 m close to the main road were the statistically significant variables with *Brucella*-DNA presence (*p*-value < 0.05). All statistically significant variables and those with *p*-value < 0.25 but considered critical to the bovine brucellosis were offered to the models (Tables 1 and 2).

The final regression model at the animal-level, five variables explained 74.2% of association with *Brucella*-DNA presence: the use of bulls for mating (OR = 2.7; 95% CI: 1.11, 6.66), pregnancy cows (OR = 1.86; 95% CI: 1.03, 3.36) reproductive problems records (OR = 3.14; 95% CI: 1.09, 8.99), parlor milking (OR

= 2.92; 95 % CI: 1.62, 5.26), belonging to farms close < 200 m from the main road (OR = 2.47; 95% CI: 1.39, 4.39). At final logistic regression model from the herd-level, two variables explained 84 % of the probability of *Brucella*-DNA presence in herds: reproductive problems records (OR = 7.76; 95 % CI: 1.17, 51.56), and the use of the bull for mating (OR = 6.49; 95 % CI: 1.20, 35.12) (Table 3).

Table 3
Multivariable models at the animal and the herd level for molecular detection of *Brucella* spp. by qPCR assay in dairy cattle of Antioquia, Colombia.

		Animal-level = 656 (*)				**Herd-level = 40 (**)**			
Factors	**Category**	**β**	**S.E (β)**	p	**OR (IC 95%)**	**β**	**S.E (β)**	p	**OR (IC 95%)**
Reproductive problems	Yes	1.14	0.537	0.033	3.14 (1.09, 8.99)	2.05	0.966	0.034	7.76 (1.17, 51.56)
	No					ref.			
Bulls	Yes	1.00	0.457	0.029					
	No	ref.			2.72 (1.11, 6.66)	1.87	0.861	0.030	6.49 (1.20, 35.12)
Parlor milking	Yes	1.07	0.301	0.000	2.92 (1.62, 5.26)				
	No	ref.							
Pregnacy status	Yes	0.622	0.302	0.039	1.86 (1.03, 3.36)				
	No	ref.							
	Yes	0.905	0.293	0.002	2.47 (1.39, 4.39)				
Proximity main road	No	ref.							
	Constant	-5.47	0.711	0.000		-2.47	1.124	0.028	

Dependent variable: positive animal, results given with β: beta; S.E: standard errors; OR: odds ratio; (95% CI): confidence interval with $p < 0.05$.

(*) The Hosmer–Lemeshow goodness-of-fit test showed that the model fit the data ($X^2 = 10.960$, d.f. 8, *p*-value = 0.20)

(**) The Hosmer–Lemeshow goodness-of-fit test showed that the model fit the data ($X^2 = 3.76$, d.f. 2, *p*-value = 0.15)

Discussion

Brucellosis is a “tool-deficient” disease that has not yet reached satisfactory levels of disease control (World Health Organization, 2007). The detection of antibodies provides only a provisional diagnosis, but there may also be an exceptionally long incubation period in some infected animals, and individuals may remain serologically negative for a considerable period following infection (Corbel, 2006). The epidemiological studies are based on the serological test to report the significant association between environmental factors, animals' intrinsic characteristics, and bovine brucellosis (Ahasan et al., 2017; Selim et al., 2019). This epidemiological study evaluated factors widely established by serology but associated them with *Brucella* DNA's presence in dairy cattle using the Probe-qPCR assay.

An unusual increase in the number of seropositive cattle during official surveillance led to the declaration of quarantine for bovine brucellosis in the leading dairy region in Colombia (Instituto Colombiano Agropecuario, 2018b). Although brucellosis is considered endemic in Latin America, there is still a need to evaluate the regional epidemiological situation (World Health Organization and Pan American Health Organization, 2016). The prevalence changes all the time in endemic countries (Godfroid et al., 2002) and it is necessary to update the epidemiological situation analysis to implement effective control and prevention strategies. This study is the first report of molecular prevalence in the region, showing a *Brucella*-DNA prevalence in concordance with the declared outbreak and higher than the seroprevalence records in the region.

Bovine whole blood as the sample for PCR detection of *Brucella* spp. has been little considered because *Brucella* may replicate at low frequency, leading to low and short bacteremia periods (Vrioni et al., 2008). However, under experimental conditions, it has been shown that some *Brucella* species could invade red blood cells and settle in the cytoplasm without replicating, establishing persistent bacteremia with different bacterial loads (Vitry et al., 2014; Gwida et al., 2016). In humans, a study of three patients infected in the recent Brucellosis outbreak in China confirmed that *Brucella* persists in peripheral blood (Baoshan et al., 2020). In this study, a high recovery of *Brucella*-DNA from whole bovine blood was possible. A high *Brucella*-DNA presence from bovine blood by qPCR, was also obtained in a bovine brucellosis outbreak study in Egypt (Gwida et al., 2016).

The *Brucella*-DNA presence could indicate the viable bacteria's existence in the cattle blood, but there is also a possibility that the presence of *Brucella*-DNA could be associated to particle shedding by dead bacteria or vaccine-DNA (Vrioni et al., 2008). The main used vaccine in the studied region is the RB51, however, the presence of the RB51 vaccine strain in the blood should not generate alerts. According to The Animal and Plant Health Inspection Service (APHIS), the RB51 must be rapidly cleared from peripheral blood within three days post-vaccination (National Council of State Legislatures, 2018). The RB51 vaccine's influence was ruled out in the positive samples by the SNP-based qPCR assay, which marked all positive samples as *Brucella* spp. field strains.

The variable municipality, mainly San Pedro de Los Milagros (SPM), showed a significant association with infection at the animal-level. SPM is considered an essential route for livestock movement, has an important cattle auction, and is close to Medellín (the second-largest city in Colombia), where the state's

central slaughterhouse and an important cattle market are located. The preceding increases the animals' passage of different origins, which may influence the region's spread of pathogens. Infected animals passing through populated areas may produce heavy contamination of streets and places (Corbel, 2006). These circumstances could have contributed to the association between the proximity of the herds to the main roads (≤ 200 m) and the presence of *Brucella*-DNA in the logistic regression model at the animal-level. Few brucellosis studies have been carried out with spatial analysis involving variables such as proximity to urban and rural areas, altitude, the season of the year, among others (Ibrahim et al., 2010; Dadar et al., 2020), and according to the authors' review, no studies in livestock have evaluated the proximity of herds to main roads. Due to the expansion of the *Brucella* genus, its evolution, and possible close relationship with environmental bacteria (Ashford et al., 2020), spatial studies are needed to understand the *Brucella* behaviour in environments.

Animals that belonged to a large herd size (> 50 animals), parlor milking, mechanical milking in pen, high grazing density (≥ 3.5 UA), an animal that belonged to herds with unknown or seropositive brucellosis status were associated with the *Brucella*-DNA presence. Dias et al. (2009) and Bugeza et al. (2019) attributed the higher risk of *Brucella* infection in large herds sizes due to the ease of transmission by the proximity between animals (Dias et al., 2009; Bugeza et al., 2019). Management practices in these farms can also influence (Moreno, 2014). Given the low numbers of *Brucella* organisms that can serve as a source of contamination, prompt detection in field samples and the reinforcement of biosecurity measures are required.

Although the bovine species are the main target of control and eradication programs, other hosts can also be infected by *B. abortus* or even by another *Brucella* spp. Some of them can sustain the disease and are considered an infection source for cattle (World Organization for Animal Health, 2018). However, in this study, *Brucella*-DNA presence was not associated with co-grazing with other animal species. Similar results were obtained in a recent epidemiological study in the country (Cárdenas et al., 2019).

In dairy herds, mainly in large herd sizes, there is a practice of raising calves and heifers in a different property from milk production. Some studies reported that animals' mobilization is a risk factor for introducing brucellosis in herds free of the disease (Cárdenas et al., 2019; Corbel 2006). Since these animals are exposed to different management practices and environmental conditions, this exposure can favor brucellosis infections (Corbel, 2006; Wareth et al., 2015). In this study, no significant association of the presence of *Brucella*-DNA with the raising of calves in other farms was found, but a significant association was found with the transfer of animals. Raising calves and heifers at a different property from the dairy cows can be appropriate if managed correctly. Separating and keeping the calves and heifers isolated from other herds, then testing them appropriately prior to their return will prevent *Brucella* introduction into dairy herds (Instituto Colombiano Agropecuario, 2017).

In the logistic regression model, the use of bulls for mating explained the *Brucella*-DNA presence at the animal and the herd level in the present investigation. Other epidemiological studies also showed a significant association of the bulls with *Brucella* seropositivity. They considered that sharing a stud bull

with neighbors could likely be a potential source of *Brucella* infection (Muma et al., 2007; Ahasan et al., 2017). *Brucella* spp. can be found in the testicles and the male genital glands and transmitted by infected semen (Corbel, 2006). However, differences in vaginal acidity, mucosal immunity, and cervical mucus between estruses and anoestrus may affect bacterial survival and kill the bacteria (Uhrig et al., 2013). Therefore, it is more likely that females' infection may occur through environmental contamination with infected seminal discharges (Thomsen, 1943). In Yellowstone National Park, USA, a study showed seroconversion in four of eight bison after intravaginal inoculation suggests that *B. abortus* transmission by the venereal route in bison can occur. Although routes of transmission are considered like those in cattle (Uhrig et al., 2013), older experiments did not find an essential role in bulls' use for mating in *Brucella* transmission in cows during copulation (Thomsen, 1943). Updated studies on venereal transmission in cattle are needed. Meanwhile, all bulls from known infected herds should be viewed with suspicion regarding their *Brucella* spp. infection status (Plant et al., 1976).

Management practices, mainly associated with reproduction, are far more critical in determining the risk of *Brucella* spp. infection in bovine (Corbel, 2006). Variables such as the history of reproductive problems, pregnant cows, days of gestation, and disposal of birth waste were associated with the *Brucella*-DNA presence in the animals evaluated. Pregnant cows have been associated with the probability of *Brucella*-antibody presence in a cross-sectional study in Ethiopia and a study conducted for isolation and subsequent detection by PCR of *Brucella* spp. in milk samples from seronegative cows in Bangladesh, even showed prevalence almost double in pregnant cows than in nonpregnant cows (Islam et al., 2018). Apart from the last two, these factors were associated with *Brucella*-DNA presence in the logistic regression models.

Conclusion

This study found the recovery of *Brucella*-DNA from whole bovine blood was possible and considered it a clinical sample for *Brucella*-DNA detection and highlighted the complementary role of PCR techniques in detection, provided a highly sensitive molecular test is used.

Permanent surveillance of brucellosis in livestock is imperative. Several epidemiological aspects obtained from the molecular prevalence evidenced in this study are in concordance with other seroprevalence studies. However, this research retakes unconsidered associations, like the bull's role in the brucellosis transmission, and suggests the need for spatial studies and transmission models in environments. The current research also highlights that management practices, mainly associated with reproduction, favors the *Brucella* infection and its propagation inside and outside herds.

Given the fluctuant brucellosis prevalence in endemic areas, updated epidemiological studies are necessary to evaluate the disease dynamic, and if established prevention and control measures have been effective or need to be adjusted.

Declarations

Funding

This work was supported by the Coordination for the Improvement of Higher Education Personnel (CAPES, finance code 001). This work was financial supported, by the National Council for Scientific and Technological Development (CNPq) for the fellowship granted to HAS (Research Productivity Scholarship, grant number 310819/2018-0) and the Foundation Carlos Chagas Filho Research Support of the State of Rio de Janeiro (FAPERJ).

Conflict of Interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Availability of data and material

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

Code availability

Not applicable

Authors' contributions

All authors contributed to the study´s conception and design. Material preparation, data collection and analysis were performed by [Olga Lucia Herrán Ramirez], [Huarrisson Azevedo Santos], [Isabele Da Costa Angelo], [Patrícia Gonzaga Paulino] and [Carolina Soares van De Meer]. The draft preparation of the manuscript was written by [Luis Rodríguez Bautista], [Ingrid Lorena Jaramillo Delgado], [Juliana González Obando] and [Rene Ramirez Garcia]. Funding acquisition, supervision [Huarrisson Azevedo Santos], and [Isabele Da Costa Angelo and all authors reviewed and edited the final version of the manuscript. All authors read and approved the final manuscript.

Ethics approval

This study involved a questionnaire-based survey of farmers as well as blood sampling from their animals. The study protocol was assessed and approved by Human Research Ethics Committee (CEP Approval number 1.243/18) from Rural Federal University of Rio de Janeiro (UFRRJ). The study have been approved by the Ethics Committee for Animal Use (CICUA Approval number AE-028/ Acta 043 301120), from CES University.

Consent to participate

Participants signed the informed consent agreement for animal blood sampling as well as for the related survey questions. Collection of blood samples was carried out by veterinarians adhering to the regulations and guidelines on animal welfare and biosecurity.

Consent for publication

Not applicable

Acknowledgements

We would like to thank the Colombian Agricultural Institute (ICA) and the Federal Rural University of Rio de Janeiro for facilitating the execution of this study. We also thanks to the corporation “Corpogansa del Norte” and their associates for allowing us to conduct the research on their cattle.

References

1. Ahasan, M. S., Rahman, M. S., Rahman, A. K. M. A., & Berkvens, D. (2017). Bovine and Caprine Brucellosis in Bangladesh: Bayesian evaluation of four serological tests, true prevalence, and associated risk factors in household animals. *Tropical Animal Health and Production*, *49*(1), 1–11. https://doi.org/10.1007/s11250-016-1151-1
2. Ashford, R., Muchowski, J., Koylass, M., Scholz, H., & Whatmore, A. (2020). Application of Whole Genome Sequencing and Pan-Family Multi-Locus Sequence Analysis to Characterize Relationships Within the Family Brucellaceae. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.01329
3. Baoshan, L., Yinbo, Y., Jingbo, Z., Yi, Z., Jianghua, Y., Dawei, C., Chi, M., Donghai, Y., Bohan, Y., Rongnian, Z., Sheng, F., Jun, Z., Han, X., & Chen, Z. (2020). Combined nucleic acid assays for diagnosis of A19 vaccine-caused human brucellosis. *Transboundary and Emerging Diseases*, tbed.13685. https://doi.org/10.1111/tbed.13685
4. Bricker, B. J. (2002). PCR as a diagnostic tool for brucellosis. *Veterinary Microbiology*, *90*(1–4), 435–446. https://doi.org/10.1016/S0378-1135(02)00228-6
5. Bugeza, J., Muwonge, A., Munyeme, M., Lasuba, P., Godfroid, J., & Kankya, C. (2019). Seroprevalence of bovine brucellosis and associated risk factors in Nakasongola district, Uganda. *Tropical Animal Health and Production*, *51*(7), 2073–2076. https://doi.org/10.1007/s11250-018-1631-6
6. Cárdenas, L., Peña, M., Melo, O., & Casal, J. (2019). Risk factors for new bovine brucellosis infections in Colombian herds. *BMC Veterinary Research*, *15*(1), 81. https://doi.org/10.1186/s12917-019-1825-9
7. Corbel, M. J. (2006). *Brucellosis in humans and animals Food and Agriculture Organization of the United Nations 92 4 154713 8*. https://www.who.int/csr/resources/publications/Brucellosis.pdf
8. Dadar, M., Shahali, Y., & Fakhri, Y. (2020). A primary investigation of the relation between the incidence of brucellosis and climatic factors in Iran. *Microbial Pathogenesis*, *139*. https://doi.org/10.1016/j.micpath.2019.103858
9. Dias, R. A., Gonçalves, V. S. P., Figueiredo, V. C. F., Lôbo, J. R., Lima, Z. M. B., Paulin, L. M. S., Gunnewiek, M. F. K., Amaku, M., Ferreira Neto, J. S., & Ferreira, F. (2009). Situação epidemiológica da

brucelose bovina no Estado de São Paulo. *Arquivo Brasileiro de Medicina Veterinaria e Zootecnia*, *61*(SUPP.1), 118–125. https://doi.org/10.1590/S0102-09352009000700015

10. Godfroid, J., Saegerman, C., Wellemans, V., Walravens, K., Letesson, J. J., Tibor, A., Mc Millan, A., Spencer, S., Sanna, M., Bakker, D., Pouillot, R., & Garin-Bastuji, B. (2002). How to substantiate eradication of bovine brucellosis when specific serological reactions occur in the course of brucellosis testing. *Veterinary Microbiology*, *90*(1–4), 461–477. https://doi.org/10.1016/S0378-1135(02)00230-4
11. Gopaul, K. K., Sells, J., Bricker, B. J., Crasta, O. R., & Whatmore, A. M. (2010). Rapid and reliable single-nucleotide polymorphism-based differentiation of Brucella live vaccine strains from field strains. *Journal of Clinical Microbiology*, *48*(4), 1461–1464. https://doi.org/10.1128/JCM.02193-09
12. Gwida, M., El-Ashker, M., Melzer, F., El-Diasty, M., El-Beskawy, M., & Neubauer, H. (2016). Use of serology and real-time PCR to control an outbreak of bovine brucellosis at a dairy cattle farm in the Nile Delta region, Egypt. *Irish Veterinary Journal*, *69*(1), 3. https://doi.org/10.1186/s13620-016-0062-9
13. Hull, N., Miller, J., Berry, D., Laegreid, W., Smith, A., Klinghagen, C., & Schumakera, B. (2018). Optimization of brucella abortus protocols for downstream molecular applications. *Journal of Clinical Microbiology*, *56*(4). https://doi.org/10.1128/JCM.01894-17
14. Ibrahim, N., Belihu, K., Lobago, F., & Bekana, M. (2010). Sero-prevalence of bovine brucellosis and its risk factors in Jimma zone of Oromia Region, South-western Ethiopia. *Tropical Animal Health and Production*, *42*(1), 35–40. https://doi.org/10.1007/s11250-009-9382-z
15. Instituto Colombiano Agropecuario. (2017). Resolución 7231 de 2017. *Instituto Colombiano Agropecuario*. https://www.ica.gov.co/normatividad/normas-ica/resoluciones-oficinas-nacionales/2017/2017r7231.aspx
16. Instituto Colombiano Agropecuario. (2018a). *Instituto Colombiano Agropecuario - ICA*. https://www.ica.gov.co/areas/pecuaria/servicios/epidemiologia-veterinaria/censos-2016/censo-2017.aspx
17. Instituto Colombiano Agropecuario. (2018b). *Resolución ICA No. 30392 de 2018*. Instituto Colombiano Agropecuario.
18. Islam, M. S., Islam, M. A., Khatun, M. M., Saha, S., Basir, M. S., & Hasan, M.-M. (2018). Molecular Detection of Brucella spp. from Milk of Seronegative Cows from Some Selected Area in Bangladesh . *Journal of Pathogens*, *2018*, 1–7. https://doi.org/10.1155/2018/9378976
19. Moreno, E. (2014). Retrospective and prospective perspectives on zoonotic brucellosis. In *Frontiers in Microbiology* (Vol. 5, Issue MAY). Frontiers Research Foundation. https://doi.org/10.3389/fmicb.2014.00213
20. Moreno, E. (2020). The one hundred year journey of the genus Brucella (Mayer and Shaw 1920). *FEMS Microbiology Reviews*. https://doi.org/10.1093/femsre/fuaa045
21. Muma, J. B., Samui, K. L., Oloya, J., Munyeme, M., & Skjerve, E. (2007). Risk factors for brucellosis in indigenous cattle reared in livestock-wildlife interface areas of Zambia. *Preventive Veterinary*

Medicine, *80*(4), 306–317. https://doi.org/10.1016/j.prevetmed.2007.03.003

22. National Council of State Legislatures. (2018). *Brucella abortus Strain RB51 Vaccine Licensed for Use in Cattle*. State Milk Laws. https://doi.org/10.1128/CVI.00326-10
23. Plant, J. W., Claxton, P. D., Jakovljevic, D., & Saram, W. (1976). Brucella abortus infection in the bull. *Australian Veterinary Journal*, *52*(1), 17–20. https://doi.org/10.1111/j.1751-0813.1976.tb05363.x
24. Queipo-Ortuño, M. I., Morata, P., Ocón, P., Manchado, P., & Colmenero, J. D. (1997). Rapid diagnosis of human brucellosis by peripheral-blood PCR assay. *Journal of Clinical Microbiology*, *35*(11), 2927–2930. http://www.ncbi.nlm.nih.gov/pubmed/9350761
25. Selim, A., Attia, K., Ramadan, E., Hafez, Y. M., & Salman, A. (2019). Seroprevalence and molecular characterization of Brucella species in naturally infected cattle and sheep. *Preventive Veterinary Medicine*, *171*. https://doi.org/10.1016/j.prevetmed.2019.104756
26. Sohn, A. H., Probert, W. S., Glaser, C. A., Gupta, N., Bollen, A. W., Wong, J. D., Grace, E. M., & McDonald, W. C. (2003). Human neurobrucellosis with intracerebral granuloma caused by a marine mammal Brucella spp. *Emerging Infectious Diseases*, *9*(4), 485–488. https://doi.org/10.3201/eid0904.020576
27. Thomsen, A. (1943). Does The Bull Spread Infectious Abort-Ion in Cattle? Experimental Studies from 1936 to 1942. *Journal of Comparative Pathology and Therapeutics*, *53*, 199–211. https://doi.org/10.1016/s0368-1742(43)80019-9
28. Trotta, A., Marinaro, M., Cirilli, M., Sposato, A., Adone, R., Beverelli, M., Buonavoglia, D., & Corrente, M. (2020). Brucella melitensis B115-based ELISA to unravel false positive serologic reactions in bovine brucellosis: A field study. *BMC Veterinary Research*, *16*(1), 50. https://doi.org/10.1186/s12917-020-02278-7
29. Uhrig, S. R., Nol, P., McCollum, M., Salman, M., & Rhyan, J. C. (2013). Evaluation of transmission of Brucella abortus strain 19 in bison by intravaginal, intrauterine, and intraconjunctival inoculation. *Journal of Wildlife Diseases*, *49*(3), 522–526. https://doi.org/10.7589/2012-03-071
30. Vitry, M. A., Mambres, D. H., Deghelt, M., Hack, K., Machelart, A., Lhomme, F., Vanderwinden, J. M., Vermeersch, M., De Trez, C., Pérez-Morga, D., Letesson, J. J., & Muraille, E. (2014). Brucella melitensis invades murine erythrocytes during infection. *Infection and Immunity*, *82*(9), 3927–3938. https://doi.org/10.1128/IAI.01779-14
31. Vrioni, G., Pappas, G., Priavali, E., Gartzonika, C., & Levidiotou, S. (2008). An Eternal Microbe: Brucella DNA Load Persists for Years after Clinical Cure . *Clinical Infectious Diseases*, *46*(12), e131–e136. https://doi.org/10.1086/588482
32. Wareth, G., Melzer, F., Tomaso, H., Roesler, U., & Neubauer, H. (2015). Detection of Brucella abortus DNA in aborted goats and sheep in Egypt by real-time PCR Veterinary Research. *BMC Research Notes*, *8*(1). https://doi.org/10.1186/s13104-015-1173-1
33. World Health Organization. (2007). Global Plan to Combat Neglected Tropical Diseases, 2008-2015. *World Health*, 37. https://doi.org/WHO/CDS/NTD/2007.3
34. World Health Organization and Pan American Health Organization. (2016). *55.º Consejo Directivo. 68.ª Sesión del Comité Regional de la OMS para las Americas*.

35. World Organization for Animal Health. (2018). Terrestrial manual: OIE - World Organisation for Animal Health. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, *2*, 1185–1191. https://www.oie.int/en/standard-setting/terrestrial-manual/

Figures

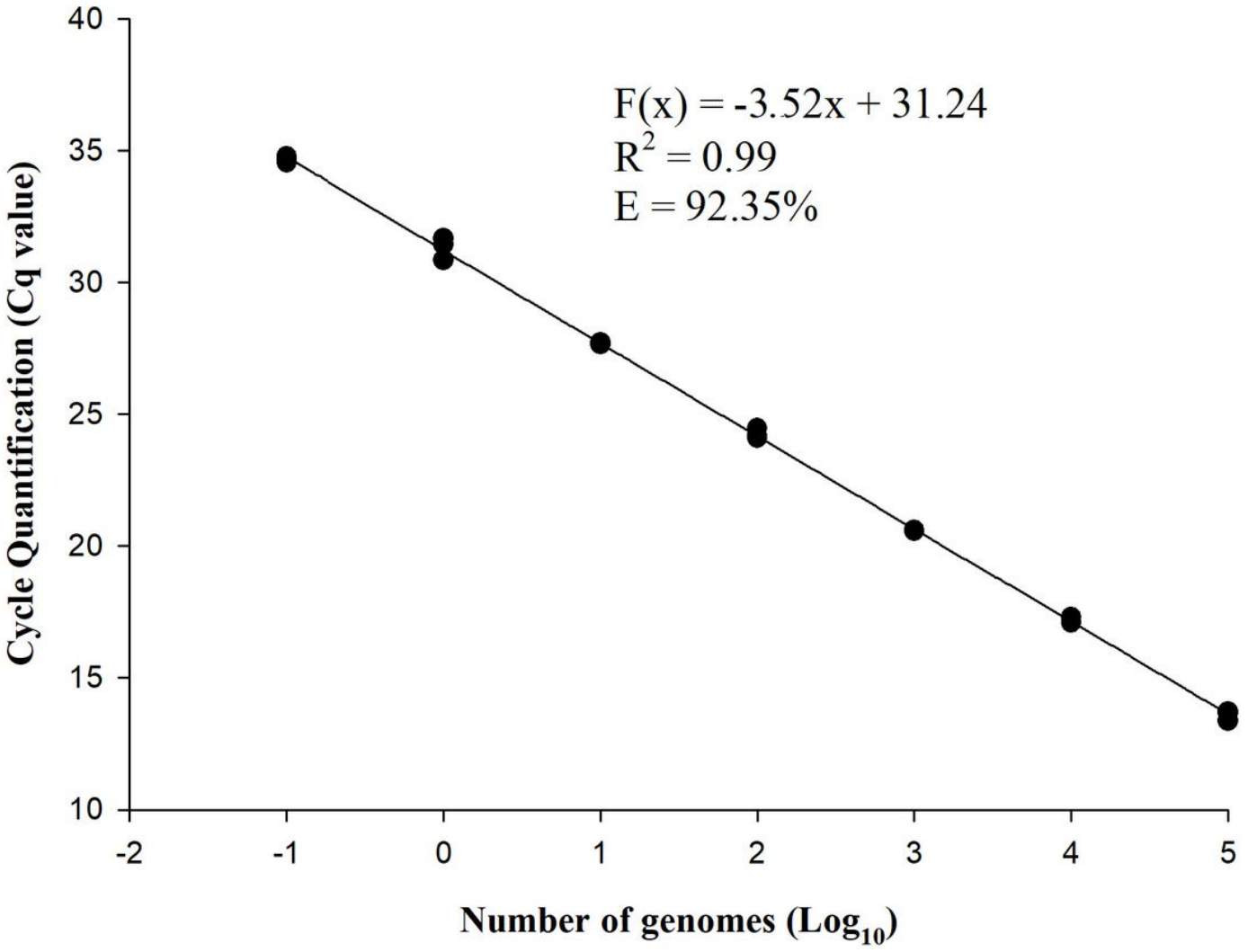

Figure 1

The standard curve was plotted using six serial decimal dilutions of Brucella abortus S19 vaccine DNA (from105 and 0.1 copies). The quantification cycle value was plotted as a function of the initial number of copies.

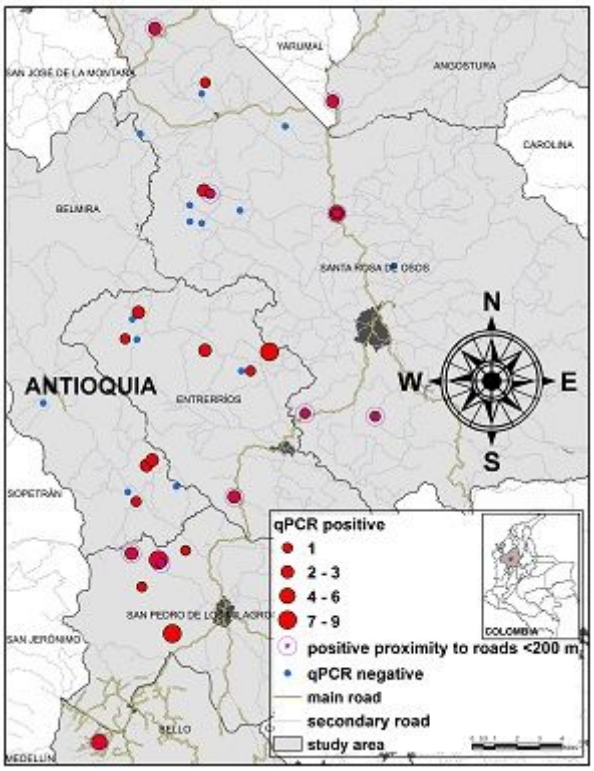

Figure 2

Spatial distribution of Brucella spp. infectious in herds, proximity to the main roads, and the number of positive cows by qPCR in the dairy region of Antioquia, Colombia. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterial.DNAExtractionProtocol.pdf