Evaluation of polymorphisms at the 3´ UTR *SLC11A1* gene microsatellites and their associations with the outcome of *Brucella abortus* infection in *Bos taurus* cattle

November 16, 2022

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ABSTRACT

In ruminants, polymorphisms in microsatellites at 3´untranslated region (3´ UTR) of the *SLC11A1* gene were associated with natural resistance to *Brucella* spp. and *Mycobacterium* spp. infection, but its relevance to prevent brucellosis is controversial in cattle. The aim of this study was to re-evaluate the role of these polymorphisms in the outcome of *Brucella abortus* infection in European bovine breeds. Initially, the presence or absence of specific antibodies against *Brucella abortus* in beef (*n*=74) or dairy (*n*=69) *Bos taurus* cattle at high risk of natural *Brucella* infection was used to identify susceptible (cases, infected) or resistant (control, non-infected) animals. Then, innate resistance to *Brucella* infection was evaluated in *B. taurus* peripheral blood monocyte-derived macrophages (MDMs) challenged with the pathogen. Finally, a bioinformatics analysis of the 3' UTR of the *SLC11A1* gene was performed to evaluate its putative functional impact on gene regulation. Fifty four (54) brucellosis positive and 89 brucellosis negative animals were genotyped for both microsatellites by multiplex PCR-capillary electrophoresis. Our results showed that the homozygous genotypes 159 and 175 for Ms1 and Ms2 respectively, previously defined as "resistant" genotypes, were the most frequent among the animal population. Independently, no association was detected between these or other polymorphisms and the absence or presence of humoral immune response against brucellosis. Moreover, no association was observed between the resistant genotype with the restricted *B. abortus*-intracellular growth phenotype in MDMs. *In silico* analysis of 3' UTR sequence predicted two canonical binding sites for transcriptional regulatory elements belonging to TEF-1 and SMAD families, but most importantly, the secondary structure of the 3'UTR remains unchanged regardless of the length of the microsatellites. Taken together, these results show no evidence of an association between the 3'UTR *SLC11A1* polymorphisms and natural resistance against brucellosis in cattle.

Keywords: bovine, resistance, brucellosis.

RESUMEN

Los polimorfismos presentes en los microsatélites de la región 3'UTR del gen SLC11A1 de los rumiantes fue asociada a la resistencia natural a la infección por Brucella spp. y Mycobacterium spp., aunque su relevancia en la preven-

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ción de la brucelosis bovina es controversial. El objetivo de este estudio fue reevaluar el rol de esos polimorfismos frente a una infección por B. abortus en bovinos de razas europeas. Inicialmente se utilizó la presencia o ausencia de anticuerpos específicos anti B. abortus en bovinos de carne (n=74) o leche (n=69) con alto riesgo de infección natural para identificar animales susceptibles (casos, infectados) o resistentes (controles, no infectados) a la infección. Posteriormente, la resistencia innata a la infección por B. abortus fue evaluada en macrófagos derivados de monocitos sanguíneos (MDMs) desafiados con la bacteria. Finalmente, se realizó un análisis bioinformático de la porción 3'UTR del gen SLC11A1 para evaluar el impacto funcional en la regulación del gen. Se genotiparon por electroforesis capilar – PCR multiplex para ambos microsatélites, 54 animales serológicamente positivos y 89 negativos a brucelosis. Nuestros resultados mostraron que los genotipos 159 y 175 para los Ms1 y Ms2 respectivamente, previamente definidos como "resistentes", fueron los más frecuentes entre la población estudiada. Independientemente de esto, no se detectó asociación entre estos u otros polimorfismos con la ausencia o presencia de respuesta inmune humoral a Brucella. Tampoco se observó asociación entre los genotipos resistentes y el fenotipo de crecimiento de B. abortus en MDMs. El análisis in silico de la secuencia 3' UTR predijo dos sitios de unión canónicos para elementos reguladores transcripcionales pertenecientes a las familias TEF-1 y SMAD, además de indicar que la estructura secundaria de esa porción génica permanecía inalterable independientemente de la extensión de los microsatélites. En conjunto, estos resultados indican una falta de asociación entre los polimorfismos en la porción 3'UTR del gen SLC11A1 y la resistencia natural a la brucelosis en los bovinos de origen europeos.

Palabras clave: *bovinos; resistencia, brucelosis.*

INTRODUCTION

Bovine brucellosis is a zoonotic disease spread worldwide, mainly caused by *Brucella abortus*. This is clinically characterized by abortions during the last trimester of pregnancy, causing damage to fertility and possibly decreased milk production. Brucellosis is transmitted to humans through consumption of unpasteurized milk or dairy products, or by direct contact with infected animals, placentas and aborted fetuses (Dean *et al*., 2012). The disease is endemic in Argentina where approximately 2,600 (40%) dairy farms and 12% (95% CI: 10% - 14%) of the 180,000 beef farms, and 0.8% (95% CI: 0.5% - 1.05%) of the 46 million cattle are infected with *B. abortus* in Argentina (Aznar *et al*., 2017).

Complex interactions of *Brucella* with environment and hosts play a critical role in the development of brucellosis. Host genetic factors, as well as innate and adaptive immune responses significantly influence the outcome of infectious diseases. In recent years, the possibility of identifying and associating polymorphisms in innate immune genes with genetic resistance to bacterial infections has begun to be considered as a complementary useful disease control strategy. Most associations with disease susceptibility lie within non-coding genomic regions, as Genome-wide association studies (GWAS) have shown; however, these associations could have functional significance, such as polymorphisms within the 3´ untranslated region (UTR) that may affect microRNA binding sites or regulatory elements that modify RNA stability, transcription and translation (Ramsuran *et al*., 2018).

The best-characterized candidate gene that controls innate resistance to intracellular pathogens in cattle is the *Solute Carrier Family 11 Member 1* (*SLC11A1*) gene, formerly known as *Natural Resistance-Associated Macrophage Protein 1* (*NRAMP1*) gene (Feng *et al*., 1996). The *SLC11A1* product is an integral membrane protein of 548 amino acids, made up of 12 transmembranes containing three phosphorylation sites, two SH3 binding motifs, and a glycosylation site. The SLC11A1 protein is found in the phagolysosomal membrane of macrophages and participates in dual functions in macrophages, contributes to the recycling of metals within the host, and to the defense of the host, by depriving ingested pathogens from micro-nutrients such as iron and manganese (Cellier, 2017).

Polymorphisms in microsatellites at 3´ UTR of the *SLC11A1* gene of the livestock species were significantly associated with resistance to *Brucella abortus* infection in water buffalo (Capparelli *et al*., 2007a,b), *B. melitensis* infection in goats (Iacoboni *et al*., 2014), *Mycobacterium bovis* infection in cattle (Kadarmideen *et al*., 2011) and *M. avium* subsp. *paratuberculosis* infection in cattle and goats (Korou *et al*., 2010; Pinedo *et al*., 2009; Taka *et al*., 2013). However, the association studies between polymorphisms in microsatellites at the 3´ UTR of the *SLC11A1* gene and resistance to *Brucella abortus* infection in cattle have yielded controversial results.

There are two polymorphic microsatellites at the 3´ UTR end of the bovine *SLC11A1* gene as a result of a variation in the number of Guanine Thymine repeats (GTn), named microsatellite 1 (Ms1) and microsatellite 2 (Ms2) (Hasenauer *et al*., 2013). For the Ms1, two previous studies have found an association between polymorphisms in this microsatellite (genotype 159/159 or GT_{12}/GT_{12}) and the control of *in vitro* replication of *Brucella abortus* by macrophage killing assay (Martinez *et al*., 2008 and 2010). However, no other studies were conducted to confirm this association. For the Ms2, a significant association was initially established between the 175/175 genotype (or GT_{13}/GT_{13}) and resistance to experimental *B. abortus* infection (Templeton and Adams, 1990) that was later correlated with increased expression of the *SLC11A1* gene and *in vitro* control replication of the pathogen (Barthel *et al*., 2001). However, two more recent reports could not confirm this association (Kumar *et al*., 2005; Paixao *et al*., 2007). Moreover, the association between the combination of Ms1 and Ms2 genotypes and control of *B. abortus* intra macrophage replication or resistance to *B.*

abortus infection in cattle was not evaluated, as well as the presence of putative regulatory motives and their functional impact in gene transcription.

Due to the scarce studies and their controversial results, we decided to re-evaluate the influence of polymorphisms in microsatellites at the 3´UTR of the *SLC11A1* gene as suitable molecular markers of resistance to *Brucella* spp. infection in cattle. Furthermore, to reinforce our research, we performed an *in silico* analysis to elucidate whether the length of polymorphic microsatellites perturbs the secondary structure of the 3'UTR that could affect the cooperative binding of transcriptional factors required for optimal gene expression.

MATERIALS AND METHODS

Case-control study design

A total of 143 unrelated female *Bos taurus* cattle from two different herds located in the central eastern region of Argentina (*the Pampas*) were included in this study (table 1). Genealogy records were not available for these herds; however, it was estimated that the analyzed cows had minimal kinship since most of them had been purchased at different auctions. All animals belonged to herds with a high prevalence of brucellosis (>30%) and clinical signs of the disease such as abortions. All animals in each herd shared the same environment for at least one year prior to sample collection for this study and were maintained under similar feeding and handling practices, suggesting that cases and controls were homogeneous in terms of environmental exposure to *Brucella* infection.

Brucellosis status was determined by serology. Serum samples (*n*=143) were firstly screened for detection of *Brucella*-specific antibodies by buffer phosphate antigen test (BPAT; sensitivity [Se] = 95.4%, specificity [Sp] = 97.7%) (Gall and Nielsen, 2004). Animals with BPAT negative serum samples were considered brucellosis free (controls), whereas BPAT positive sera were confirmed by standard tube agglutination test (STAT; Se = 75.9%, Sp = 95.7%) and 2- mercaptoethanol (2-ME; Se = 88.4%, Sp = 91.5%) performed in parallel as described by Alton *et al*. (1988). The interpretation of the results was made following the procedures recommended by SENASA (National Office of Animal Health), Argentina. Only those animals positive for BPA and with titers ≥ 1:200 to SAT and/or ≥ 1:25 to 2-ME test were considered brucellosis positive (cases).

In vitro infection of peripheral blood monocytes-derived macrophages with *B. abortus*

Peripheral blood monocyte-derived macrophages (MDMs) for *in vitro* infection assays were isolated from eight healthy six-month-old female *Bos taurus* calves from the experimental herd of the Research Center for Veterinary and Agronomical Sciences (CICVyA) of the National Institute for Agricultural Technology (I.N.T.A.), following the protocol previously published (Rossetti *et al*., 2011). Animals were non-vaccinated against *B. abortus* and serologically negative to *Brucella* spp.-specific antibodies. Briefly, after 10 days in culture, MDMs from each animal were challenged in triplicate with *B. abortus* S19 at the multiplicity of infection (MOI) ratio of 5:1 (bacteria: MDMs). After 1h incubation (37ºC in 5% CO_2) to allow bacteria: MDMs interaction, extracellular bacteria were killed by replacing the culture media in each well by RPMI-gentamicin medium (37.5 µg/ml final concentration; Sigma, St. Louis, MO), and one hour later, the cultures were washed three times with sterile PBS to remove the antibiotic. To determine the number of phagocytosed *B. abortus* S19 (T0) or intracellularly viable S19 (T24), MDMs monolayers were lysed with 100µl of 0.5% Tween-20 (Sigma) in sterile distilled water immediately after washing or after 24h post-infection, respectively. The lysates were serially diluted in sterile water and plated in duplicates on trytose-soy agar (TSA) for CFU quantification. Intracellular *B. abortus* S19 survival (rSOB24h) was estimated as the square root of the number of CFUs at T24 relative to the number of CFUs at T0 {rSOB24h = √[(T24/T0)x100]}. MDMs with restrictive or permissive intracellular *B. abortus* survival and replication phenotype present rSOB24h <10 o ≥10, respectively, that correlate with resistant (restrictive) or susceptible (permissive) *B. abortus* infection phenotypes (Martinez *et al*., 2010). Duplicate wells containing single bacterial suspensions were plated and used as a control for the adequacy of antibiotic killing of extracellular bacteria and for control of bacterial growth (>1log in 24h). *In vitro* infection assays were done twice per animal.

DNA extraction and microsatellites genotyping

All the animals included in this study were genotyped for both microsatellites in the 3' UTR of the bovine *SLC11A1* gene (GenBank U12862) by multiplex PCR-capillary electrophoresis. Genomic DNA was isolated from hair follicles using the phenol: chloroform: isoamyl alcohol protocol as previously reported (Hasenauer *et al*., 2013).

Ms1 was amplified using primers Fw1 5´-GATCAGGAGAAGGGGAGGA-3´ and Rv1 5´-CAGCTTCCAGAACTCCCTGT-3´ (nucleotide positions 1691 to 1848) whereas Ms2 was amplified using primers Fw2 5´-AAGGCAGCAAGACAGACAGG-3´ and Rv2 5´-ATGGAACTCACGTTGGCTG-3´ (nucleotide positions 1814 to 1988) (Hasenauer *et al*., 2013). The Fw1 primer was 5´ labeled with the fluorescent dye NED whereas the Rv2 primer was labeled with 6-carboxyfluorescein. Multiplex PCR was carried out in 15 µl containing 1X reaction buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.2666 µM of the primers Fw1 and Rv1 and 0.0533 µM of the primers Fw2 and Rv2, 0.5 units of Taq DNA polymerase (Inbio-Highway, Tandil, Argentina) and 20 ng of genomic DNA. Amplification reactions were performed with an initial step of 5 min at 94°C; 40 cycles of 45s at 94°C, 30s at 59°C, 45s at 72°C; and a final extension step of 60 min at 72°C. PCR products were separated by capillary electrophoresis on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystem, Waltham, MA) as previously published (Hasenauer *et al*., 2013), and the results were analysed with GeneMapper software (Applied Biosystems). To confirm the length of Ms1 and Ms2 (GT repeats), the PCR products of three animals homozygous for the identified alleles were sequenced in both directions.

Based on previous findings (Hasenauer *et al*., 2013; Martinez *et al*., 2008 and 2010; Templeton and Adams, 1990), 159/159 bp (or GT_{12}/GT_{12}, Ms1), 175/175 bp (or GT_{13}/GT_{13}, Ms2) or 159/159-175/175 (Ms1 and 2) at the 3´UTR *SLC11A1* gene were defined as "resistant" ("R") (animal) or "restrictive" ("Re") (MDMs) genotypes, while non-159/159 bp, non-175/175 bp or non-159/159 – non-175/175 were considered "susceptible" ("S") (animal) or "permissive" ("P") (MDMs) genotypes.

Bioinformatic analysis

In silico analysis of the 3´UTR *SLC11A1* bovine gene (GenBank U12862) was performed by RegRNA 2.0 software (http://RegR-

NA.mbc.nctu.edu.tw/) (Chang *et al*., 2013) using a *Bos taurus* database. The RNA secondary structures for R and S alleles were predicted using RNAfold WebServer (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) (Gruber *et al*., 2008).

Statistical analysis

Allelic and genotypic frequencies and HardyWeinberg equilibrium were calculated using GenePop software (Raymond and Rousset, 1995). The detection of significant associations between the absence or presence of *Brucella*-specific antibodies (controls and cases, respectively) and polymorphisms at the 3´ UTR *SLC11A1* gene microsatellites (resistant and susceptible genotypes) were verified by Fisher's exact test using GraphPad software (on-line version: http://www.graphpad.com/quickcalcs/). Odd´s ratio (OR) and 95% confidence intervals (95% CI) were calculated using VassarStats (http://vassarstats.net/odds2x2.html). Significance was set at $p \leq 0.05$.

RESULTS

Serology

Brucella-specific antibodies were detected by BPAT and confirmed later by SAT and 2-ME in 25 and 29 animals in Bos *taurus* beef cattle and dairy herds, respectively (table 1). These 54 serologically positive cows were considered cases (brucellosis positive); in contrast, the other 89 animals serologically negative to brucellosis were controlled.

Microsatellites

Genotyping by capillary electrophoresis displayed the presence of four genotypes (157/157, 157/159, 159/159, 159/161) for the Ms1, and two genotypes (175/175, 175/177) for the Ms2 (table 2). Alleles 159 (or GT_{12}, for the Ms1) and 175 (or GT_{13}, for the Ms2) were the most frequent in both infected and non-infected cattle (fig. 1).

Cases and controls were screened separately for the deviation of genotype distribution from the Hardy-Weinberg equilibrium. The genotype frequencies of both microsatellites (Ms1 and Ms2) at the 3' UTR of the bovine *SLC11A1* gene were in Hardy-Weinberg equilibrium ($p>0.05$) between controls and cases in beef cattle, dairy cattle and the entire population.

Transcription regulatory motifs

An *in silico* analysis predicted two canonical binding sites for transcriptional regulatory elements. A consensus TEF-1 (translation elongation factor-1) recognition motif, also known

Herd	Region	Number of animals analyzed	Serologically positive animals (BPAT; SAT/2-ME)	Prevalence of brucellosis (%)
Beef cattle	Hughes, Santa Fe (33°48'04"S 61°20'10"W)	74	25	33,78
Dairy cattle	Vedia, Buenos Aires (34°29'44"S 61°32'20"W)	69	29	42,03
Total		143	54	37,76

Table 1. Sample size and serology results.
Analysis of the 3'UTR of *SLC11A1* bovine gene

Genotypes at 3´ UTR *SLC11A1*	Beef cattle			Dairy cattle			Total		
	Frequency	Serological results		Frequency	Serological results		Frequency	Serological results	
		Positive	Negative		Positive	Negative		Positive	Negative
Microsatellite 1									
157/157 (GT_{10}/GT_{10})	0,013	1	0	0,044	2	1	0,028	3	1
157/159 (GT_{10}/GT_{12})	0,257	5	14	0,304	11	10	0,28	16	24
159/159 (GT_{12}/GT_{12})	0,689	18	33	0,652	16	29	0,671	34	62
159/161 (GT_{12}/GT_{13})	0,041	1	2	0	0	0	0,021	1	2
Microsatellite 2									
175/175 (GT13/GT13)	0,946	24	46	1	29	40	0,972	53	86
175/177 (GT13/GT14)	0,054	1	3	0	0	0	0,028	1	3

Table 2. Genotype frequencies at the 3´ UTR SLC11A1 microsatellites in Bos taurus cattle and its relationship with serological positive or negative results to Brucella infection.

as TEAD1, was identified at nucleotide positions 1747 to 1752 (5'-GGAATG-3'); enhanson GT-IIC) (Jiang *et al*., 2000), 28 bp before the polymorphic Ms1. Moreover, a SMAD family responsive motif was identified at nucleotide position 1823 to 1831 (5'-AGACAGACA-3') (Itoh *et al*., 2019), between the Ms1 and Ms2 (fig. 2). No other regulatory elements, such as microRNA or AU-rich elements were predicted at the 3'UTR of the bovine *SLC11A1* gene.

Association of microsatellites variants with anti-*B. abortus*-specific antibodies

No significant associations were detected between microsatellite polymorphisms (at the genotype and allele level) at 3'UTR of *SLC11A1* gene with the presence or absence of anti-*Brucella*-specific antibodies (table 3). Otherwise, the secondary structure of the 3'UTR remains unchanged regardless of the length of the microsatellites, which eliminates the possibility that the modification in the secondary structure affects the cooperative binding of transcriptional regulatory elements and, consequently, the expression of the *SLC11A1*.

Evaluation of *B. abortus*-intracellular growth phenotype in bovine MDMs with resistant genotype

To evaluate in depth the role of 3'UTR of *SLC11A1* polymorphisms, we assessed the bacterial intracellular survival assay in bovine MDMs from cattle with different genotypes. Screening of a total female beef *Bos taurus* calves, non-vaccinated against *B. abortus*, from the experimental CICVyA-INTA herd (n = 80), revealed that all animals had the same genotype, i.e., homozygous 159 (GT_{12}) and 175 (GT_{13}) or "R" genotype, for both microsatellites Ms1 and Ms2, respectively. Therefore, an

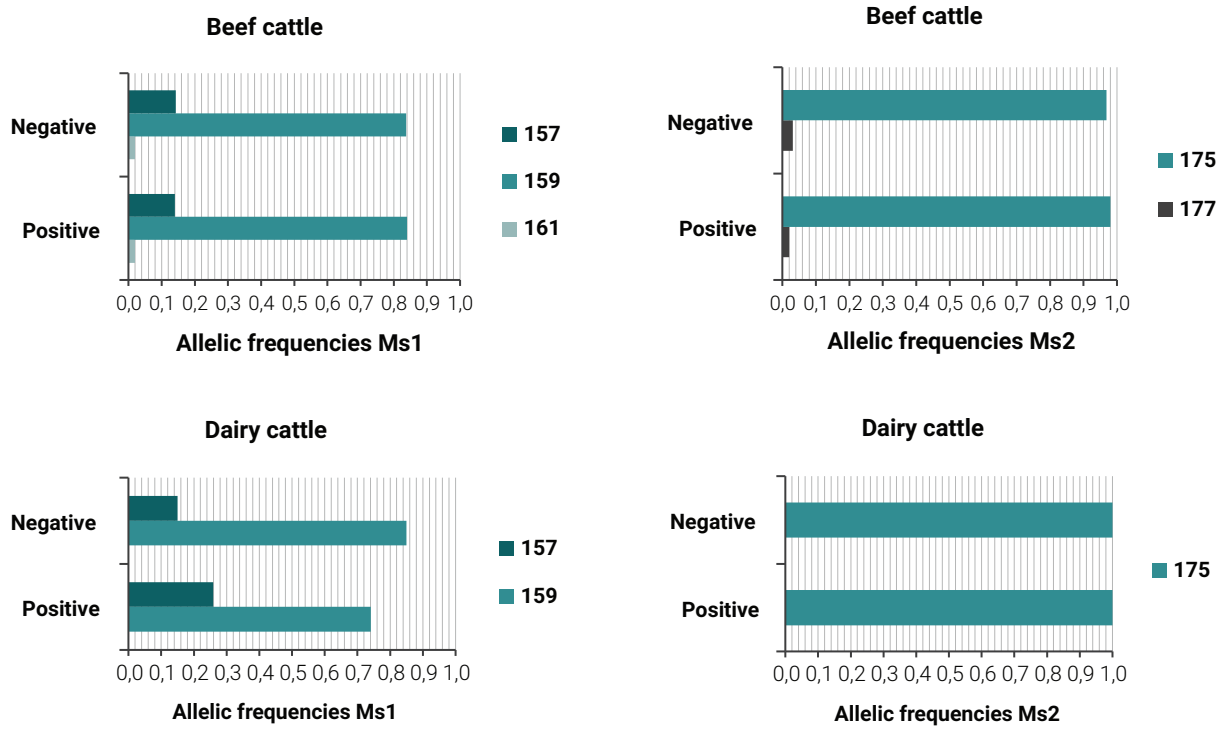

Figure 1. Allelic frequencies of the microsatellite 1 (Ms1) and microsatellite 2 (Ms2) at the 3´ UTR *SLC11A1* gene in *Bos taurus* beef and dairy cattle and its relationship with serological positive or negative results to *Brucella* infection.

gctcccacca gggcctggcc acgggt**ggaa tg**agtgggca cagtggcctg tcagacaagg

gtgtgtgtgt gtgtgtgtgt gtgtatgtgt gtgaaggcag caagacagac agggagttct

ggaagctggc caacgtgagt tccagaggga cctgtgtgtg tgtgacacac tggcctgcca

gacaaggtg tgtgtgtgtg tgtgtgtgtg tgtgcatgca cagcaagacg gagagggagt

tctggaaggc agccaacgtg agttccatag ggacctgcta tttcctagct cagatctcag

Figure 2. Sequence of the 3'UTR of the bovine *SLC11A1* gene. The figure shows the sequence of the 3'UTR *SLC11A1* gene (5'–3', position 1,721–2,020) (GenBank U12862). Microsatellite 1 is highlighted in light grey and microsatellite 2 in dark grey. The binding sites of the putative transcriptional regulatory elements are shown in bold (TEF-1) and underlined (SMAD) on the gene sequence.

Genotypes at 3´ UTR SLC11A1	Beef cattle			Dairy cattle			Total		
	Serological results			Serological results			Serological results		
	Positive	Negative	*p* value OR (95% CI)	Positive	Negative	*p* value OR (95% CI)	Positive	Negative	*p* value OR (95% CI)
Microsatellite 1									
Resistant[a]	18	33	*p* = 0.793	16	29	*p* =0.200	34	62	*p* = 0.464
Susceptible[b]	7	16	OR = 1.247	13	11	OR = 0.467	20	27	OR = 0.740
Total	25	49	(0.433-3.590)	29	40	(0.170-1.280)	54	89	(0.363-1.511)
Microsatellite 2									
Resistant[c]	24	46	*p* = 1.000	29	40	*p* = 1.000	53	86	*p* = 1.000
Susceptible[d]	1	3	OR = 1.565	0	0	OR = NaN	1	3	OR = 1.849
Total	25	49	(0.154-15.870)	29	40	(NaN)	54	89	(0.187-18.238)

Table 3. Association between resistant or susceptible genotypes at the 3´ UTR *SLC11A1* microsatellites in *Bos taurus* cattle and serological positive or negative results to *Brucella* infection. No significant differences were observed (p>0.05) based on Fisher exact tests, odd´s ratio (OR) and 95% confidence intervals (95% CI).
Reference: [a] Resistant genotype: 159/159 (or GT12/GT12); [b] Susceptible genotype: non 159/159; [c] Resistant genotype: 175/175 (or GT13/GT13); [d] Susceptible genotype: non 175/175. NaN: Not A Number.

ID animal	T0 (CFU)	T24 (CFU)	rSOB24h
	Mean (SD)	Mean (SD)	
288	7083 (1234)	14750 (2165)	14,44
292	1075 (250)	25300 (7037)	48,51
295	4800 (308)	42525 (2298)	29,75
296	832 (259)	11617 (1378)	37,38
299	8708 (2805)	20833 (7552)	15,47
300	1655 (423)	26500 (8552)	40,07
303	3200 (909)	9600 (3426)	17,32
304	5000 (1135)	19700 (7701)	19,85

Table 4. Results of B. abortus S19 killing assay on MDMs of female calves, unvaccinated against brucellosis, with restrictive genotype.

association study between "Re" or "P" MDMs genotype with MDMs phenotype could not be carried out. Independently, we randomly selected eight female calves and assessed whether the GT_{12}-GT_{13} homozygous genotype (for Ms1 and Ms2, respectively) confers a restrictive *B. abortus* intracellular survival phenotype and therefore, resistance to brucellosis. Our results showed that all MDMs analyzed presented a permissive phenotype (i.e., rSOB24h >10), and consequently an association with the "Re" or "R" genotype could not be established (table 4).

DISCUSSION

Here, we re-evaluated the association of polymorphisms at the 3´UTR *SLC11A1* microsatellites with the natural resistance of *Bos taurus* cattle to *Brucella* infection, as a consequence of contradictory results in previous studies. To do this, we conducted a case-control study in two herds with a high prevalence of natural *B. abortus* infection, and *in vitro* macrophage killing assay. The results presented here showed a lack of association between natural resistance to *Brucella* infection (i.e., absence of *Brucella*-specific antibodies and/or restrictive *B. abortus* intracellular growth in MDMs) with some molecular marker in both microsatellites (Ms1 and Ms2) at the 3' UTR of the *Bos taurus SLC11A1* gene.

Case-control studies are useful in establishing a correlation between exposures and outcomes; but to obtain reliable conclusions, studies must be conducted under stringent guidelines (Dupépé *et al*., 2019). Accurate identification of cases and controls is a fundamental step for detecting true associations in a population-based case-control study (Anonymous, 1999). The manner that the case-control population was selected in this study was similar than in other associative studies. Previous case-control studies have detected an association between intracellular pathogen infections and polymorphism at the 3' UTR of the *SLC11A1* gene in water buffalo (Capparelli *et al*., 2007a,b) and goats (Iacoboni *et al*., 2014; Korou *et al*., 2010); and the absence or presence of specific antibodies has been considered a valid indicator of "R" or "S" phenotypes, respectively. In agreement, the results of the brucellosis serological tests were considered reliable indicators to define "R" or "S" phenotypes in human case-control studies (Ismael *et al*., 2018; Keramat *et al*., 2019; Sepanjnia *et al*., 2015). Here, we also classified animals as "infected" and "non-infected" based on serological test results, as positive serology against *Brucella* spp. is a strong indicator of infection (Gall and Nielsen, 2004). In some circumstances cross-reacting antibodies could arise due to other infectious microorganisms, such as *Yersinia enterocolitica* O:9 or *E. coli* O:157 and interfere with the serological diagnosis (Mac Millan, 1990). The serological reaction to these pathogens is primarily an IgM response that is inhibited by the neutralizing effect of 2-ME on IgM (Garin Bastuji *et al*., 1999). In addition to serological results, a recent history of abortion at the last trimester of gestation had strongly indicated the presence of *B. abortus* infection in both herds. On

the contrary, serologically negative animals were considered phenotypically "R" to *Brucella* infection, since they had similar chances of becoming infected with *Brucella* spp. than the cases, although no humoral immune response was detected. The lack or weak humoral immune response to *Brucella* infection in natural resistant animals has been previously reported as a consequence that they would have the ability to induce a Th1 rather than Th2 immune response (Capparelli *et al*., 2007b; Harmon *et al*., 1985; Rossetti *et al*., 2011). Furthermore, cases and controls were well matched for sex (females), age (> 3 years) and risk of exposure to brucellosis.

The highest frequency of the 159/159 genotype in Ms1 found in this study was in agreement with our previous results in *Bos taurus*, where the 159 homozygous frequency was 0,651 in Holstein, 0.927 in Creole, 0.649 in Hereford, and 0.894 in Angus breeds (Hasenauer *et al*., 2013). These results were also in accordance with Martinez *et al*. (2010) that found 0.963 and 1.00 of 159/159 genotype frequency in Colombian Blanco Orejinegro (BON) Creole cattle and Holstein X BON crosses, respectively. Previous associative studies for the Ms1 with R or S to brucellosis linked the genotype 159/159 with natural resistance to *B. abortus* infection in cattle (Martinez *et al*., 2008 and 2010). One of these studies (Martinez *et al*., 2010) reported that cows with a homozygous 159 genotype had a significant greater inhibition of the anti-antibody response after *B. abortus* S19 challenge, and their MDMs had a higher restriction of *B. abortus* intramacrophagic growth in an *in vitro* killing assay than cows with other genotypes. Contrary to these results, in our hands, the homozygous 159 genotype of Ms1 could not be associated with the restrictive phenotype of MDMs or resistance to *Brucella* infection under natural conditions.

The Ms2 175/175 genotype that was correlated with the homozygous 13 GTs repeats was found at a high frequency (> 0.95) in the *Bos taurus* herds analyzed in this study. The low variability of this molecular marker in *Bos taurus* cattle in Argentina has been previously reported by our research group where the frequency was 0.963 in Holstein, 0.976 in Creole, 0.947 in Hereford, and 0.915 in Angus (Hasenauer *et al*., 2013). Similar results were shown by others in Colombia and in Brazil who found a homozygous GT_{13} genotype in 100% of the Holstein cattle analyzed (Gonzalez *et al*., 2006; Paixao *et al*., 2006). The relationship between homozygous 175 genotype with natural resistance to *B. abortus* infection was detected for the first time in a group of crossbred cattle (*B. taurus* x *B. indicus*) by experimental challenge (Templeton and Adams, 1990) and was later correlated in an *in vitro* assay of *B. abortus* intracellular growth (Barthel *et al*., 2001). In contrast, in more recent studies, resistance to brucellosis could not be associated with this genotype in *B. indicus* and *B. taurus* x *B. indicus* crossbred (Kumar *et al*., 2005; Paixao *et al*., 2007). Kumar *et al*. (2005) found that all the cattle included in the study (infected and non-infected), had the "R" genotype (GT_{13} / GT_{13} or 175/175), while Paixao *et al*. (2007) observed a high frequency of homozygous GT_{13} genotype in both cases (0.84) and control (0.86) groups analyzed. In concordance with those authors, our results showed a lack of association between "R" genotype and the absence of *Brucella* infection or restriction to *B. abortus* intracellular replication in MDMs of *B. taurus*. Initially, our goal was to evaluate and eventually correlate the MDMs phenotype ("Re" and "S") with the "R" and "S" genotype, but the absolute frequency of "R" genotype precluded our original goal. The reasons why a high percentage of *B. taurus* has the homozygous "R" genotype at the 3'UTR of *SCL11A1* gene (Ms1 and 2) could be due to prolonged selective breeding or *Bos taurus* inbreeding. Although it could also be related to other elements, such as increased fertility, milk production, postnatal survival under particular environmental conditions, or decreased susceptibility to pathogens other than *Brucella* spp.

SLC11A1 resistant associated genotype to *Brucella* infection is attributable to a higher rate of transcription or mRNA stability mediated through the length of the 3'UTR poly (GT) repeated sequence (Barthel *et al*., 2001 Capparelli *et al*., 2007b). It is well known that the length of microsatellites may influence the regulation of gene expression through changes in the α-helical phase and therefore affect the cooperative binding of transcription factors necessary for optimal expression of the gene (Bagshaw, 2017). For the first time, we have performed a bioinformatic analysis of the 3'UTR of *SLC11A1* bovine gene looking for regulatory elements that bind in this region. Our analysis found two canonical binding sites for transcriptional regulatory elements, and most importantly, the secondary structure of the 3'UTR remains unchanged regardless of microsatellite length. These data would eliminate the possibility that the modification in the secondary structure affects the cooperative binding of transcriptional regulatory elements and influences the expression of the *SLC11A1* and, consequently, the innate response against *Brucella* infection.

In the light of these and previous results, natural resistance to brucellosis would likely be determined by the actions of various interacting genes rather than being perfect for the GT polymorphisms at the 3'UTR of the *SLC11A1* bovine gene (Templeton and Adams, 1990). In support of this, it was observed that susceptible (BALB/c) and resistant (C57B1/10) mouse models of brucellosis carried the *SLC11A1* susceptible allele (Skamene *et al*., 1982). It is also important to mention that the infection process is influenced by host factors including genetic background, imprinting effect and species-specific susceptibility. For instance, one study showed that MDMs from *B. indicus* (Zebu) were more efficient at controlling intracellular replication of *B. abortus* than MDMs from *B. taurus* (Holstein) cattle (Macedo *et al*., 2013), while other study reported opposite results (Martinez *et al*., 2008). In that study, Martinez *et al*. (2008) found significant differences in the intracellular number of *B. abortus* in MDMs derived from BON x Zebu compared to MDMs collected from Zebu x BON crossbred at 24 and 48h post-infection. Furthermore, differences in experimental designs, protocols and execution of the experiments from different research groups (which include the degree of MDMs maturation, positive and negative controls, biological and technical replicas, the time post-infection in which the intracellular CFU is determined, etc), sample size, genetic background of the animal population, statistical analysis, and some other reasons could explain the discrepancies in the results and their subsequent interpretation of associative studies between polymorphisms at 3' UTR bovine *SLC11A1* gene and the phenotype to *Brucella* infection.

CONCLUSION

Association between polymorphisms previously identified as resistant in Ms1 (GT12-GT12; 159/159) and/or Ms2 (GT13-GT13; 175/175) at the 3' UTR of *SLC11A1* gene and the absence of *Brucella*-specific antibodies such as natural resistance parameter against *B. abortus* infection in cattle, was not found in this study. Moreover, no association was observed between the

resistant genotype with the restricted *B. abortus*-intracellular growth phenotype in MDMs. The lack of association reported here does not mean that the *SLC11A1* gene product plays no role in modulating and controlling the innate response to intracellular bacterial pathogens, as many other publications indicate. Undoubtedly, future studies aimed at elucidating the role of *SLC11A1* gene in phenotyping resistance against *Brucella* in cattle should be conducted under widely accepted standardized protocols to resolve current controversies.

ACKNOWLEDGEMENTS

The authors want to thank the animals' owners for their uninterested collaboration and Mr. Diego Franco and Mr. Claudio Fioroni for their help with the animals of the experimental herd at CICVyA-INTA. This study was funded by INTA (Instituto Nacional de Tecnología Agropecuaria), Grant PNBIO 1131033. Student stipend support provided by CONICET (Argentina) (FCH).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

FCH, MAP and CAR conceived and designed the study. FCH and MEC performed the experiments and analyzed the data. FCH wrote the original draft. CAR and MAP reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

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