Veterinary Immunology and Immunopathology 160 (2014) 230–234

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Polymorphisms at the 3′ untranslated region of *SLC11A1* gene are associated with protection to *Brucella* infection in goats

Paola A. Iacoboni[a], Flavia C. Hasenauer[a,e], M. Eugenia Caffaro[b], Analia Gaido[c], Cristina Rossetto[d], Roberto D. Neumann[c], Antonio Salatin[c], Emiliano Bertoni[c], Mario A. Poli[b], Carlos A. Rossetti[a,*]

[a] *Instituto de Patobiología, CICVyA-CNIA, INTA, Nicolas Repetto y de Los Reseros s/n, Hurlingham, Buenos Aires, Argentina*
[b] *Instituto de Genética Edwald A. Favret, CICVyA-CNIA, INTA, Nicolas Repetto y de Los Reseros s/n, Hurlingham, Buenos Aires, Argentina*
[c] *EEA Salta, INTA, RN 68, km 172, Cerrillos, Salta, Argentina*
[d] *INTA EECT Yuto, RN 34, km 1286, Yuto, Jujuy, Argentina*
[e] *Conicet, Buenos Aires, Argentina*

ARTICLE INFO

Article history:
Received 8 November 2013
Received in revised form 4 April 2014
Accepted 13 May 2014

Keywords:
SLC11A1 gene
Goats
Brucellosis
Innate resistance

ABSTRACT

Goats are susceptible to brucellosis and the detection of *Brucella*-infected animals is carried out by serological tests. In other ruminant species, polymorphisms in microsatellites (Ms) of 3′ untranslated region (3′UTR) of the solute carrier family 11 member A1 (*SLC11A1*) gene were associated with resistance to *Brucella abortus* infection. Goats present two polymorphic Ms at the 3′UTR end of *SLC11A1* gene, called regions A and B. Here, we evaluated if polymorphisms in regions A and/or B are associated with *Brucella* infection in goats. Serum (for the detection of *Brucella*-specific antibodies) and hair samples (for DNA isolation and structure analysis of the *SLC11A1* gene) were randomly collected from 229 adult native goats from the northwest of Argentina. Serological status was evaluated by buffer plate antigen test (BPAT) complemented by the fluorescent polarization assay (FPA), and the genotype of the 3′UTR of the *SLC11A1* gene was determined by capillary electrophoresis and confirmed by sequence analysis. Polymorphisms in regions A and B of the 3′UTR *SLC11A1* gene were found statistically significant associated with protection to *Brucella* infection. Specifically, the association study indicates statistical significance of the allele A_{15} and B_7/B_7 genotype with absence of *Brucella*-specific antibodies ($p = 0.0003$ and 0.0088, respectively). These data open a promising opportunity for limiting goat brucellosis through selective breeding of animals based on genetic markers associated with natural resistance to *B. melitensis* infection.

1. Introduction

Goats are susceptible to brucellosis, a worldwide zoonotic infectious disease that has a significant economic impact in livestock industry and human public health. Caprine brucellosis is mainly caused by *Brucella melitensis* and it is clinically characterized by temporal infertility and middle to late gestation abortion in pregnant females, and orchitis in males (Alton, 1990). Although caprine brucellosis has been controlled in most industrialized countries, it remains a major problem in the Mediterranean region, the Middle East, Central Asia, sub-Saharan Africa, and Latin

* Corresponding author. Tel.: +54 11 4621 1289; fax: +54 11 4621 1289.
E-mail address: rossetti.carlos@inta.gob.ar (C.A. Rossetti).

http://dx.doi.org/10.1016/j.vetimm.2014.05.007
0165-2427/

Table 1
Sample size and serology results.

Farm number	Number of samples (serum and hair)	Positive animals (BPAT and FPA)	Prevalence of brucellosis (%)
1	18	7	39
2	14	4	29
3	22	7	32
4	23	7	30
5	12	6	50
6	13	3	23
7	34	17	50
8	17	4	24
9	32	5	15
10	15	14	93
11	16	11	69
12	13	3	23
Total	229	88	38

America (FAO, 2010). For practical, large-scale surveillance the detection of *Brucella*-infected animals is carried out by serological tests (Gall and Nielsen, 2004).

The solute carrier family 11 member A1 (*SLC11A1*) gene, formerly known as natural resistance-associated macrophage protein 1 (*NRAMP1*) gene, has been identified as the candidate gene controlling innate resistance to intracellular pathogens. In ruminant species, polymorphisms in microsatellites (Ms) of 3′ untranslated region (3′UTR) of the gene were associated with resistance to *Brucella abortus*, *Mycobacterium bovis* and *Mycobacterium avium* subsp. Paratuberculosis (Barthel et al., 2001; Borriello et al., 2006; Capparelli et al., 2007a; Kadarmideen et al., 2011; Korou et al., 2010; Martinez et al., 2008, 2010; Pinedo et al., 2009; Reddacliff et al., 2005; Taka et al., 2013). There are two polymorphic Ms in the 3′UTR end of the caprine *SLC11A1* gene as a result of a variation in the number of guanine-thymine variation repeats $(GT)_n$ (Vacca et al., 2011). The upstream Ms (named region A) showed to be more polymorphic than the downstream Ms (named region B), where only two alleles were reported (Korou et al., 2010; Vacca et al., 2011).

The goal of this study was to evaluate a potential association between polymorphisms in region A and/or B of the 3′UTR *SLC11A1* gene and the presence or absence of *Brucella*-specific antibodies in goats.

2. Materials and methods

2.1. Sample collection

A total of 229 serum and hair samples were randomly collected from adult female creole crossbreed goats from 12 goat farms located in Rivadavia county, Salta Province, in the northwest of Argentina (Table 1). The region is characterized by a semiarid climate with a mean annual temperature of 22 °C and a mean annual rainfall of 500 mm. Animals included in the present study were unvaccinated against brucellosis and belonged to flocks with a high prevalence of brucellosis (>30% of the animals were serologically positive for brucellosis) and clinical signs such abortion (Gaido et al., 2011). They were all equally exposed to *Brucella* infection. The serum samples were used for the detection of *Brucella*-specific antibodies whereas the hair samples were used for DNA isolation and structure analysis of regions A and B of the 3′UTR *SLC11A1* gene.

2.2. Detection of Brucella-specific antibodies

Blood samples were obtained by jugular puncture; following centrifugation, the sera were separated and stored at −20 °C until used. Serum samples ($n = 229$) were firstly screened for detection of *Brucella*-specific antibodies by buffer plate antigen test (BPAT). Briefly, 80 μl of serum and 30 μl of antigen (Instituto de Sanidad Ganadera, CABA, Argentina) were mixed with a spreader on a glass plate and incubated for 8 min at room temperature. The plate was hand-rotated three times at 4 min after mixing to ensure a uniform suspension of reagents, and return for a second 4-min incubation. Well known positive and negative goat serum were used as controls. Positive or negative results were determined by the presence or absence, respectively, of visible agglutination. The test had a 98.1% of sensitivity and 97% of specificity. Positive BPAT sera were confirmed by the fluorescence polarization assay (FPA). FPA was performed with sera samples diluted 1:25 in 10–75 mm glass tubes. A baseline evaluation of serum samples fluorescence polarization level was obtained using FP Sentry 1000 (Diachemix, Wiscosin, USA) and subsequently 10 μl of antigen labeled with fluorescein isothiocyanate (FITC) (Laboratorio Biológico de Tandil, Tandil, Argentina) was added to each tube and mixed well. After two minutes of incubation, the tubes were read again, and the results were expressed in milipolarization units (mP). FPA cut off value was established in >85 mP with a sensitivity of 94.9% and specificity of 99.4%. Positive and negative sera were included for control of test performance. Both tests were performed according to the Argentinean National Veterinary Services and the OIE recommendations (OIE, 2008; SENASA, 2009). For association study, brucellosis positive or negative animals were considered those with BPAT and FPA positive, or BPAT negative results, respectively. Animals with BPAT positive not confirmed by FPA were not included in the analysis.

2.3. DNA isolation and genotyping

Hair samples were stored in a paper envelope at room temperature. Genomic DNA was isolated from hair follicles following the protocol previously published (Hasenauer et al., 2013) with minor adaptations for this experiment. Briefly, thirty to forty 96% ethanol-cleaned hair follicles were collected into a 1.5 ml microtube containing 200 μl of SNET lysis buffer (20 mM Tris–HCl, pH 8.0; 5 mM EDTA; 400 mM NaCl; 1% w/v SDS) and 4 μl of proteinase K (Fermentas, Vilnius, Lithuania). After being incubated for 45 min at 55 °C followed by 15 min at 95 °C, 120 μl of the supernatant were transferred to a clean 1.5 ml tube, followed by an addition of 24 μl of sodium chloride (5 mM) and 150 μl of phenol:chloroform:isoamyl alcohol (Invitrogen, Carlsbad, CA). After centrifugation at 800 × *g* for 10 min, the aqueous phase was transferred to a clean 1.5 ml microtube, and mixed with 220 μl of absolute ethanol. The DNA pellet was recovered by centrifugation at 12,000 × *g* for 20 min after being washed twice with 70% ethanol. The

DNA pellet was air-dried and then resuspended in 30 μl of TE buffer. DNA samples' concentration was quantified by NanoDrop® ND-1000 (NanoDrop, Wilmington, DE) and stored at −20 °C until used.

Multiplex PCR was carried out to amplify both Ms at the 3′UTR of the caprine SLC11A1 gene (GenBank FJ388877). Primer forward 1 (Fw1; 5′-GTCTGGACCTGTCTCATCACC-3′), reverse 1 (Rv1; 5′-ACTCCCTCTCCATCTTGCTG-3′) and reverse 2 (Rv2; 5′-AAGTGCTAGGCTCTGCGTTC-3′) had been previously described (Vacca et al., 2011). Primer forward 2 (Fw2; 5′-ATGAGTGGGCACAGTGGC-3′) was designed using Primer3 software (Rozen and Skaletsky, 2000). Primer pair 1 and primer pair 2 amplified a region of approximately 230 bp containing region A, and 202 bp containing region B, respectively. Primers Fw1 and Rv2 were 5′ labeled with the fluorescent dye 6-FAM (6-carboxyfluorescein) and VIC, respectively. Multiplex PCR was carried out with 30 ng of genomic DNA in a total reaction volume of 15 μl containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM of each primer and 0.5 U of Taq DNA polymerase (Inbio-Highway, Tandil, Argentina). Amplification reactions were performed with an initial denaturation step of 6 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 45 s at 63 °C and 1 min at 72 °C, with a final extension step of 15 min at 72 °C. PCR products were evaluated in a 1.5% agarosa gel electrophoresis stained with ethidium bromide.

Genotyping was performed by capillary electrophoresis as previously described (Hasenauer et al., 2013), and the size of the alleles was determined using GeneMapper software version 4 (Applied Biosystems). To confirm the length of regions A and B (GT repeats), PCR products from at least three homozygous animals for the identified alleles were sequenced in both directions. The nucleotide sequence was determined using version 3.1 of the Big Dye terminator cycle sequencing kit (Applied Biosystems) and the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The sequence data were analyzed using BioEdit (Hall, 1999).

2.4. Statistical analysis

The statistical significance of the association ($p < 0.05$) between the polymorphisms in region A and/or B of the 3′UTR end of the caprine *SLC11A1* gene and the absence of *Brucella*-specific antibodies in the serum was determined by the Fisher' exact test using GraphPad software (on-line version: http://www.graphpad.com/quickcalcs/).

3. Results

3.1. Serology

The prevalence of brucellosis in analyzed animals was between 15 and 93% (Table 1). *Brucella*-specific antibodies were detected first by BPAT and confirmed later by FPA, in 38% of the analyzed serum (88 of 229). Four samples were disregarded from further analysis since they were BPAT positive but not confirmed by FPA.

Table 2
Genotype frequencies at the 3′UTR *SLC11A1* microsatellites in Argentinean native goats and its relationship with *Brucella*-specific antibodies.

Region	Frequency	Serological results	
		Positive	Negative
A			
A_{13}/A_{13}	0.031	3	4
A_{13}/A_{14}	<0.01	–	1
A_{13}/A_{15}	<0.01	–	2
A_{13}/A_{16}	0.105	13	11
A_{13}/A_{17}	0.013	–	3
A_{13}/A_{18}	0.04	4	5
A_{14}/A_{14}	<0.01	1	–
A_{14}/A_{15}	<0.01	1	–
A_{14}/A_{16}	0.017	3	1
A_{14}/A_{17}	<0.01	–	1
A_{14}/A_{18}	<0.01	–	1
A_{15}/A_{15}	0.017	–	4
A_{15}/A_{16}	0.105	3	21
A_{15}/A_{17}	0.017	1	3
A_{15}/A_{18}	0.07	4	12
A_{16}/A_{16}	0.205	17	29
A_{16}/A_{17}	0.08	9	9
A_{16}/A_{18}	0.165	20	17
A_{17}/A_{17}	<0.01	2	–
A_{17}/A_{18}	0.026	1	5
A_{18}/A_{18}	0.06	6	8
B			
B_7/B_7	0.16	7	29
B_7/B_8	0.47	46	60
B_8/B_8	0.37	35	48

3.2. Microsatellites at the 3′UTR of SLC11A1 gene analysis

The number of GT variation repeats found in region A ranged from 13 to 18, being six alleles identified [A_{13}:(GT_{13}), A_{14}:(GT_{14}), A_{15}:(GT_{15}), A_{16}:(GT_{16}), A_{17}:(GT_{17}), A_{18}:(GT_{18})] and 21 genotypes. Only two alleles [B_7:(GT_7) and B_8:(GT_8)] and three genotypes were present in region B (Table 2).

3.3. Association of Brucella-specific antibodies and 3′UTR alleles

The association analysis between serological results and polymorphisms in region A and/or B of the 3′UTR end of the caprine *SLC11A1* gene indicates statistically significant association of the allele A_{15} and the B_7/B_7 genotype with absence of *Brucella*-specific antibodies ($p = 0.0003$ and 0.0088, respectively) (Table 3A and B). The two sided p value computed by Fisher exact test indicates that there would be less than 0.1% and 1% chances respectively of randomly picking animals with so much association if the A_{15} or B_7/B_7 genotype and the absence of *Brucella*-specific antibodies were not associated. There were no statistical significant association between the absence of *Brucella* antibodies and B_7 allele (i.e., $B_7/B_7 + B_7/B_8$ genotypes; $p = 0.48$) or any other allele found in Region A.

4. Discussion

Detection of antibodies against *Brucella* sp. in unvaccinated adult goats by accurate serological diagnosis tests is a strong indicator of *Brucella* infection (Gall and Nielsen,

Table 3
Association of the serological test results with the genotypes of the region A (A) or region B (B) at the 3′UTR end of *SLC11A1* gene. Statistical significance was considered for $p < 0.05$ based on Fisher's Exact Test.

(A)		
Genotype region A	Serological results	
	Positive	Negative
$A_{15}/A_{15} + A_{15}/A_n$	9	42
A_n/A_n	79	95
(B)		
Genotype region B	Serological results	
	Positive	Negative
B_7/B_7	7	29
$B_7/B_8 + B_8/B_8$	81	108

A_n = any A allele but A_{15}.
$p = 0.0003$.
$p = 0.0088$.

2004). The results presented here show a significant association between the allele A_{15} (15 GTs variation repeats in region A) in heterozygous or homozygous condition, and the genotype B_7/B_7 at the 3′UTR of *SLC11A1* caprine gene with absence of *Brucella*- specific antibodies. Korou et al. (2010) found that the presence of B_7 allele in homozygous or heterozygous condition was significantly associated with absence of *M. avium* subsp. Paratuberculosis (MAP)-specific antibodies in goats, but they did not find association between absence of MAP antibodies with polymorphisms in region A. In this study, the presence of the B_7 allele in heterozygous condition was not sufficient to avoid the presence of *Brucella*-specific antibodies ($p = 0.48$). Similar studies in other ruminant species had also established an association between a particular *SLC11A1* genotype and the lack or presence of *Brucella* infection determined by specific antibodies. For instances, the BB genotype in water buffalo was significantly associated with negative results to serological test of brucellosis, while the *SLC11A1* AA genotype was associated with susceptibility to *Brucella* infection (Borriello et al., 2006; Capparelli et al., 2007a, 2007b). In parallel, the homozygous genotypes CC from SPN4 and AA from SPN5 of *SCL11A1* gene were more frequent in *Brucella* serologically positive cows than in serologically negative animals (Paixao et al., 2012).

Lack or significantly lower amount of *Brucell*a specific antibodies in A_{15}/A_n or B_7 homozygous goats could be partially explained by the fact that *SLC11A1* gene is significantly higher expressed in resistant than in susceptible animals (Barthel et al., 2001; Capparelli et al., 2007a) which would induce a strong Th1 immune response. In previous studies was observed that mice or mouse macrophages carrying wild type *Nramp1* allele (i.e. *Nramp1*^{G169}, resistant animals) challenged with intracellular pathogens, mounted a Th1 immune response and expressed higher levels of pro-inflammatory cytokines (IL-1B, IL-6, IL-12, TNFα) than animals or cell mouse with mutant *Nramp1*^{G169D} gene (Soo et al., 1998; Valdez et al., 2008). It is well known that Th1 cellular immune response is effective to promote *Brucella* clearance from the host (Dornand et al., 2002; Oliveira et al., 2002; Rolán and Tsolis, 2008) while Th2 humoral immune response is detrimental for controlling *B. abortus* infection (Fernández and Baldwin, 1995). In agreement with these observations, Rossetti et al. (2011) showed that resistant animal to *B. abortus* infection had the ability to mount a Th1 immune response against this pathogen that was impaired in susceptible animal. In the same direction, Harmon et al. (1985) demonstrated that after an experimental challenge with *B. abortus*, cattle resistant to *B. abortus* infection developed low transient serologic titers and were negative for *Brucella* isolation, while susceptible infected cows developed high serologic titers, aborted and *Brucella* was isolated from secretions. Altogether, these data suggest that *SLC11A1* A_{15}/A_n or *SLC11A1* B7 homozygous animals would not present *Brucella*- specific antibodies after *Brucella* infection because they would have the ability to induce a Th1 instead of Th2 immune response. In order to understand why few *SLC11A1*- A_{15}/A_n or B_7 homozygous goats presented *Brucella*-specific antibodies, we can speculate that these antibodies would belong to a transient immune response of a recent *B. melitensis* infection; or as expected for a multigenic trait, the association of natural resistance was not perfect for the GT polymorphisms of *SLC11A1* (Adams and Schutta, 2010). Undoubtedly, further studies are needed to unravel *SLC11A1* regulation after intracellular pathogen infection and how the gene expression interacts with host immune response.

The frequency of the allele A_{15} found in this study was much lower than the frequency reported for this allele among Greek goats, although higher than observed in Italian goats (12% in this study vs. 34% and 3%; Table 2) (Korou et al., 2010; Vacca et al., 2011). On the other hand, the allele frequency in region B of the 3′UTR of the *SLC11A1* gene was similar to that observed by Korou et al. (2010) (40% allele B_7 and 60% allele B_8 in this study vs. 44 and 56%, respectively; Table 2); however, the percentage of goats with B_7/B_7 genotype found among animals evaluated by us was lower than those reported in other goats breeds (16% in this study vs. 26%) (Korou et al., 2010; Taka et al., 2013). The reasons why few percentage of northwest Argentinian native goats presents a B_7 homozygous genotype may be linked to other elements, like reduced fertility, milk production or postnatal survival under particular environmental conditions, or increase susceptibility to pathogens other than *B. melitensis*, and it is something that worth to find.

Goats' world current population is over 900 million animals with 90% of them located in developing countries (FAO, 2010). Caprine brucellosis control and eradication measures consist on test-and-slaughter program combined with Rev1 vaccination (Blasco, 2010). However, test and slaughter is expensive to implement when flocks have high prevalence of brucellosis, while vaccine sometimes failed to protect flocks, can interfere with the serological tests, is risky for the operator and can cause abortion in females. Therefore, this study presents more results confirming the relationship between genetic markers associated with natural resistance to *Brucella* sp. infection and open a promising opportunity to use them in a selective breeding program that could be added along to other classical brucellosis control and eradication measures, to help of limiting the spread of brucellosis.

Conflict of interest statement

The authors confirm that they have no conflicts of interest in this work.

Acknowledgements

The authors are grateful to Mr. Modesto T. Giménez ("Chacho") for helping us during farm work. We also want to thank to the animals' owners for their uninterested collaboration. This study was supported by the Agencia Nacional de Promoción Científica y Tecnológica de Argentina (Grant PICT 2010-536) and INTA (Grant PE 234022).

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