Cytokine 115 (2019) 109–115

Contents lists available at ScienceDirect

Cytokine

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

Association of an *IRF3* putative functional uORF variant with resistance to *Brucella* infection: A candidate gene based analysis of InDel polymorphisms in goats

Ursula A. Rossi[a], Flavia C. Hasenauer[a,c], María E. Caffaro[b], Maria A. Raschia[b], Estefania Maurizio[a], Hector S. Cortez[d], Roberto D. Neumann[d], Mario A. Poli[b], Carlos A. Rossetti[a,*]

[a] Instituto Nacional de Tecnología Agropecuaria, CICVyA, Instituto de Patobiología, Nicolás Repetto y de Los Reseros s/n, Hurlingham, Buenos Aires B1686, Argentina
[b] Inst. de Genética "Ewald A. Favret", Nicolás Repetto y de Los Reseros s/n, Hurlingham, Buenos Aires B1686, Argentina
[c] CONICET, Buenos Aires, Argentina
[d] Instituto Nacional de Tecnología Agropecuaria, IIACS, Area de Salud Animal, RN 68 (km 172) Cerrillos, Salta, Argentina

ARTICLE INFO

Keywords:
Brucellosis
Genetic resistance
Polymorphisms
IRF3
KALRN
TIRAP

ABSTRACT

Brucellosis is an important zoonotic disease caused by infection with *Brucella* spp. It generates major economic losses in livestock production worldwide. Goats are the principal hosts of *B. melitensis*, the main infection agent of caprine and human brucellosis. The selection of resistance-related genes is considered one of the best long-term means to improve control to bacterial infection in domestic ruminants. We performed a candidate gene association study to test if six short insertion/deletion polymorphisms (InDels) at bacterial-infection related genes influence the resistance to *Brucella* infection in female creole goats. InDels (*IRF3*-540: rs660531540, *FKBP5*-294: rs448529294, *TIRAP*-561: rs657494561, *PTPRT*-588: rs667380588, *KALRN*-989: rs667660989 and *RAB5a*-016: rs661537016) were resolved by PCR-capillary electrophoresis in samples from 64 cases and 64 controls for brucellosis. Allelic frequencies were significantly different between cases and controls at *IRF3*-540 and *KALRN*-989 ($p = 0.001$ and 0.005). Indeed, the minor alleles (a and k) at InDels *IRF3*-540 and *KALRN*-989 were more frequent among controls than cases, providing evidence that these alleles confer protection against *Brucella* infection. Moreover, *IRF3*-540 a-containing genotypes (Aa and aa) were associated with absence of *Brucella*-specific antibodies in goats ($p = 0.003$; OR = 3.52; 95% CI = 1.55–7.96), and more specifically, a-allele was associated with resistance to *Brucella* infection in a dose-dependent manner. Also, we observed that the *IRF3*-540 deletion (a-allele) extends a conserved upstream ORF by 75 nucleotides to the main ORF, and thus it may decrease gene expression by reducing translation efficiency from the main ORF. These results suggest a potential functional role of *IRF3*-540 deletion in genetic resistance to *Brucella* infection in goats.

1. Introduction

Human brucellosis is one of the most frequent zoonotic infections worldwide, with approximately 500,000 new cases per year [1]. Transmission of brucellosis from animals to humans occurs mainly by direct contact with infected animals, or consumption of contaminated foods. Otherwise, brucellosis causes important economic losses in the livestock industry due to premature culling of infected animals, vaccination cost, and effects on productivity. Brucellosis in goats is predominantly caused by *Brucella melitensis*, which is considered the most pathogenic *Brucella* species for humans [2]. This disease is characterized by orchitis in male goats and reproductive failure and reduced milk production in females. Although caprine brucellosis has been controlled in many industrialized countries, it remains a major problem in most developing countries, where goats constitute one of the main sources of meat and milk production [3]. Control of caprine brucellosis relies on strategies that have considerable financial cost and are not practical or effective to eradicate the disease in developing countries. For instance, test-and-slaughter programs or massive vaccination campaigns, have contributed to the elimination of brucellosis in developed countries, but this strategy is not useful in high-prevalence settings where economic resources and veterinary services organization are not available for its support [4].

Strong evidence indicates that genetic factors influence the resistance to *Brucella* infection in livestock animals [5]. Thereby, genetic selection for disease resistance may be an effective long-term mean to

* Corresponding author.
E-mail address: rossetti.carlos@inta.gob.ar (C.A. Rossetti).

https://doi.org/10.1016/j.cyto.2018.11.024
Received 9 June 2018; Received in revised form 6 November 2018; Accepted 19 November 2018
Available online 23 November 2018

enhance resistance to brucellosis in goats, improve milk and meat safety, and reduce economic loss. To identify genetic variants involved in resistance to caprine brucellosis, we conducted a candidate gene study in a case-control sample of 128 female goats from herds with high prevalence of brucellosis. Based on a literature review, we selected six candidate genes (*IRF3*, *FKBP5*, *PTPRT*, *TIRAP*, *KALRN* and *RAB5a*) that, even being non typical candidate genes for brucellosis, they were previously linked to *Brucella* spp. and other bacterial infections. Interferon regulatory factor 3 (*IRF3*) mediates the induction of IFNβ in response to *B. abortus* and *M. tuberculosis* in mice [6,7], and an *IRF3* intronic variant was implicated in susceptibility to *Listeria monocitogenes* infection in a mice model [8]. On the other side, *FKBP5* and *PTPRT* were associated with resistance to bovine tuberculosis and caprine brucellosis [9–11]. Otherwise, single nucleotide polymorphisms (SNPs) at the Toll-like receptor adaptor (*TIRAP*) gene were associated with tuberculosis susceptibility in humans [12,13]. It is known that the *Brucella* protein TcpB suppresses host innate-immune responses by targeting TIRAP for degradation, and that TIRAP affects intracellular replication of *B. melitensis* [14,15]. The Rho GEF kinase Kalirin (*KALRN*) and the small GTPasa Rab5 (*RAB5a*) are involved in intracellular trafficking. An intronic SNP of *KALRN* was associated with increased resistance to *Mycobacterium bovis* in cattle [11], and it was reported that Rab5 plays an important role in the early phagocytic trafficking and intracellular survival of *Brucella abortus* [16,17].

This study identified two InDels (*IRF3* rs660531540 and *KALRN* rs667660989) that were significantly associated with absence of *Brucella* specific antibodies in goats. Additionally, *in silico* analysis predicted a functional role of the *IRF3* InDel in the regulation of *IRF3* mRNA translation, suggesting the presence of functional association. These results contribute to the identification of the genetic mechanisms that underlie the resistance against *Brucella* infection.

2. Materials and methods

2.1. Samples

A total of 128 unrelated female creole cross-breed goats from three unrelated flocks in the northwest region of Argentina were used (22, 23 and 19 controls and cases from flocks 1, 2 and 3 respectively). All animals were unvaccinated against *Brucella* spp. and belonged to flocks with a high prevalence of brucellosis (40%, 47% and 37% of the animals of flocks 1, 2 and 3 respectively, were serologically positive for brucellosis) and clinical signs of the disease such as abortion. Thereby, cases and controls were uniformly exposed to *Brucella* spp. Cases were animals seropositive for brucellosis according to the buffer plate antigen (BPA) screening test (98.1% SE, 97%SP) and the confirmatory fluorescence polarization assay (FPA; 94.9% SE, 99.4% SP); while controls were animals seronegative by the BPA test. Tests were performed as previously explained [18] with well known positive and negative goat control serum.

2.2. DNA isolation

Genomic DNA was isolated from hair follicles. Briefly, 150 µl of lysis buffer (20 mM Tris HCl, pH 8.0; 5 mM EDTA; 400 mM NaCl; 1% w/v SDS) and 3 µl of proteinase K (19.6 mg/ml, TermoFisher, Carlsbad, CA) were added to a 1.5 ml microtube containing thirty to forty hear follicles. After incubation for 30 min at 55 °C, 120 µl of the resulting solution was transferred to a clean microtube. Then, 24 µl of sodium chloride (5 mM) and 150 µl of phenol:chloroform:isoamyl alcohol (TermoFisher) were added, gently mixed and centrifuged (10 min, 800*g*). The upper phase was transferred to a 1.5 ml tube and two volumes of ethanol (100%) were added. After an overnight incubation at −20 °C, the precipitated DNA was collected by centrifugation (30 min, 9.000*g*), washed twice with 70% ethanol, air dried at room temperature and re-suspended in 30 µl of TE buffer. Quality and quantity of DNA samples were estimated by NanoDrop® ND-1000 (NanoDrop, Wilmington, DE).

2.3. Polymorphism selection

Using the NCBI dbSNP database, one polymorphic InDel for each candidate gene was selected based on its location in relation to the gene structure, summarized as *GENE*-InDel (NCBI SNP ID number): *IRF3*-540 (rs660531540), *FKBP5*-294 (rs448529294), *TIRAP*-561 (rs657494561), *PTPRT*-588 (rs667380588), *KALRN*-989 (rs667660989) and *RAB5a*-016 (rs661537016). *IRF3*-540 and *RAB5a*-016 are 5′ and 3′ UTR variants. *FKBP5*-294, *PTPRT*-588 and *TIRAP*-561 are intronic variants lying within or nearby regions that were previously implicated in resistance to *Mycobacterium* spp. infection. *KALRN*-989 is an intronic variant located 123 nucleotides upstream of *KALRN* exon 20.

2.4. Genotyping

InDel regions were amplified using the M13-tailed primer method [19]. PCR was carried out with 37.5 ng of genomic DNA in a total reaction volume of 15 µl containing 1X PCR buffer, 1.5 mM MgCl2, 200 µM dNTPs, 0.2 µM of M13-forward primer, 0.3 of reverse primer, 0.3 of 6-carboxyfluorescein-M13 (6-FAM-M13) primer and 0.75 U of Taq DNA polymerase (Inbio-Highway, Tandil, Argentina). Cycling conditions were: 5 min at 95 °C, followed by 35–40 cycles of 45 s at 95 °C, 30 s at 55 °C and 45 s at 72 °C, with a final step of 10 min at 72 °C. PCR products were resolved by capillary electrophoresis using the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The length of the capillary was 36 cm and the separation medium was POP-7 polymer (Applied Biosystems). The electrophoresis data was analyzed using GeneMapper software (Applied Biosystems). Allele identifications were confirmed by DNA sequencing.

Primer pairs were designed from GenBank sequences using Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/): forward primer (fw) 6-FAM-5′-GTTTTCCCAGTCACGACGTTG-3′ (6-FAM-M13); fw 5′-M13-ATCCTTCTCTCCCGAGATCC-3′ and reverse primer (rv) 5′-AACCACGATCAAGCCCTTTT-3′ (*IRF3*-540); fw 5′-M13-GGCTGGTTTTCAGCTTGATGC-3′ and rv 5′-TCCTCTACAAGGTAGCAA GTGT-3′ (*FKBP5*-294); fw 5′-M13-CGTACTCTACACCCAGCTTAG-3′ and rv 5′-CGTGTATGCTGTCAGGGATG-3′ (*TIRAP*-561); fw 5′-M13-GGAAG AATGTGGCCTGAACA-3′ and rv 5′- TAGCTCCCTCTGTTCAATAGGA-3′ (*PTPRT*-588); fw 5′-M13-TCTCCCTGATCTTGCTCTCCT-3′ and rv 5′-GTCTTGGTCATCTAGGCCTCT-3′ (*KALRN*-989); and fw 5′-AGTAAC AACCTTGCAGCCTT-3′ and rv 5′-GATTTCAGGTAACACAATGCCA-3′ (*RAB5a*-016).

2.5. Statistical analyses

Allelic and genotype frequencies were calculated with Excel 2010. Deviations from the Hardy–Weinberg equilibrium were tested using the genetic calculation javascript by Knud Christensen (http://www.husdyr.kvl.dk/htm/kc/popgen/genetik/applets/0.htm). Associations between the absence of *Brucella*-specific antibodies and individual InDels were evaluated with the Fisher's exact test using GraphPad online software (http://www.graphpad.com/quickcalcs/). Odds ratios (OR) and 95% confidence intervals (95% CI) were determined with VassarStats (http://www.vassarstats.net/odds2x2.html). In order to correct for multiple comparisons, Bonferroni-adjusted p-values of < 0.008 were considered statistically significant [20].

Logistic regression analysis under additive model was performed using SPSS Statistics (IBM Corp., Armonk, NY); p-values < 0.05 were considered significant. Hosmer – Lemeshow test was used to confirm goodness of fit of the additive model ($p = 0.856$).

2.6. DNA sequencing and LD analyses

We performed DNA sequencing of *KALRN* exon 20 and *TIRAP* exon 5 to identify SNPs that were in linkage disequilibrium with *KALRN*-989 and *TIRAP*-561 InDels, respectively. Three seropositive and three control samples of each genotype were used. PCR was carried out with 75 ng of genomic DNA in a total reaction volume of 30 µl containing 3.5% DMSO, 1X PCR buffer, 1.5 mM MgCl2, 200 µM dNTPs, 0.2 µM of each primer and 0.75 U of Taq DNA polymerase (Inbio-Highway, Tandil, Argentina). Amplification reactions were performed as described above, except that annealing temperature was 58 °C. PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and purified using ADN puriprep-GP kits (Inbio-Highway). Then, the purified products were sequenced using the Big Dye terminator cycle sequencing kit (Applied Biosystems) and the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The primers used for amplification were fw 5′-M13Fw-GGAGCAAGGCCTATGATGTG-3′ and rv 5′-M13Rv-GGCCGTCCACGAAGTACA-3′ (TIRAP exon 5); fw 5′-M13Fw-TGATCTTGCTCTCCTCTGGG-3′ and rv 5′-M13Rv- TTCTTCTC ACCCCCATCTCC-3′. The sequencing primers were 5′-TGTAAAACGAC GGCCAGT-3′ (M13Fw) or 5′CAGGAAACAGCTATGAC-3′ (M13Rv). Pairwise linkage disequilibrium as r^2 was estimated using Haploview [21].

2.7. In silico study

Regulatory RNA motifs were identified using RegRNA 1.0 software (http://regrna.mbc.nctu.edu.tw). ESE Finder (rulai.cshl.edu) was used to predict splicing signals. An InDel was predicted to have functional significance when different patterns for a regulatory motive were observed between alleles. The upstream ORF (uORF) motives and main ORF (mORF) location was confirmed by the UTRscan software (http://itbtools.ba.itb.cnr.it/utrscan).

2.8. Sequence and structure conservation analysis of IRF3 5′ UTR

The uORFs structure of caprine, ovine, bovine and human *IRF3* 5′UTR were analyzed using UTRscan. Local nucleotide alignments were performed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). *IRF3* mRNA sequences were obtained from the GenBank database: XM_013971473.2 (caprine), XM_012157808.2 (ovine), XM_015458163.1 (bovine) and NM_001571.5 (human).

2.9. In vitro infection of monocyte-derived macrophages (MDMs) with B. melitensis

Blood samples were collected from fourteen previously *IRF3*-540 genotyped unrelated goats from Buenos Aires Province, Argentina. Animals included in this study were unvaccinated and serologically negative to brucellosis by the BPA test. Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Histopaque-1077 (Sigma, St. Louis, MO), and seeded in T25 flasks in complete RPMI 1640 medium (C-RPMI) (RPMI 1640 medium – Invitrogen, Carlsbad, CA – with 2 mM L-glutamine, 1 mM non-essential amino acids, 1 mM sodium pyruvate and 2.9 mM 7.5% sodium bicarbonate) (Gibco, Life Technologies, Grand Island, NY) supplemented with 4% fetal bovine serum (FBS) (Internegocios S.A., Mercedes, Buenos Aires), 1X penicillin-streptomycin antibiotic solution (Gibco) and 0.25 µg/ml amphotericin B antimycotic (Gibco). After an overnight incubation at 37 °C in a humidified atmosphere with 5% CO_2, the flasks were washed with phosphate-buffered saline (PBS) to remove non-adherent cells, and fresh C-RPMI medium supplemented with 12% FBS and antibiotic and antimycotic solution. Cells were maintained in culture for eight days until monocytes differentiate into macrophages. Twenty-four hours prior to infection, MDMs were detached by incubation with trypsin-EDTA 1X (Sigma Aldrich), counted in a hemocytometer and seeded in triplicate into 96-bottom flat well plates at the concentration of 5×10^4 cells/well. MDMs were infected with *Brucella melitensis* 16 M at a multiplicity of infection of 10:1 (bacteria: MDMs), centrifuged (800*g* for 10 min), and replaced to the incubator for 30 min. Bacterial suspension from each well was replaced with 100 µl of C-RPMI 1640 medium supplemented with gentamicin antibiotic solution (50 µg/ml, Sigma) to kill extracellular bacteria. After 1 h incubation, the cells were gently washed with sterile PBS to remove the antibiotic and fresh medium added. Immediately (T0) or 24 h after incubation (T24), MDMs were lysed with 100 µl of 0.5% Tween 20 (Sigma) and serial dilution were plated on tryptic soy agar media for quantification of colony-forming units (CFU). Results were analyzed using two-way ANOVA followed by Bonferroni's test; p-values of < 0.01 were considered statistically significant.

3. Results

3.1. Allelic association study

Minor allele frequencies (MAFs) for six InDel polymorphisms (*IRF3*-540, *FKBP5*-294, *TIRAP*-561, *PTPRT*-588, *KALRN*-989 and *RAB5a*-016) were analyzed in 64 *Brucella*-seronegative (controls) and 64 *Brucella*-seropositive (cases) creole goats (Table 1). The genotype frequencies of all studied variants were consistent with Hardy-Weinberg equilibrium (HWE) among controls ($p > 0.008$, Table 1). The six InDels were polymorphic in all three flocks, showing total MAFs between 0.02 and 0.43. Minor allele frequencies of InDels *IRF3*-rs540 and *KARLN*-rs989 were significantly different between cases and controls ($p \leq 0.008$, Table 1), suggesting an association between the minor alleles (a and k) and absence of *Brucella*-specific antibodies.

3.2. Genotypic association study

The association of InDel genotypes with the *Brucella*-specific antibodies status (positive or negative) was determined by the Fisher's exact test (Table 2). While the *FKBP5*-294, *PTPRT*-588, and *RAB5a*-016 genotypes were not associated with the *Brucella*-specific antibodies status, the minor allele genotypes of *IRF3*-540 (Aa and aa) were strongly associated with increased resistance against *Brucella* spp. infection, compared with the AA-genotype ($p = 0.003$). Otherwise,

Table 1
Minor allele frequencies (MAF) and Hardy–Weinberg equilibrium (HWE) for the six studied InDels. Minor alleles and significant p-values are indicated in bold.

INDEL (GENE-marker)	Accession number	Location (Chr: pb)	Alleles	HWE (p-values)		MAF			
				Control	Cases	Total	Control	Cases	p-value
IRF3-rs540	rs660531540	18:54494504	[CTCCTTGCTA/–]	0.124	0.284	0.43	0.53	0.33	$\mathbf{1.5 \times 10^{-3}}$
FKBP5-rs294	rs448529294	23:9267653	[(A)7/8/**9**]	0.251	0.706	0.02	0.03	0.01	3.7×10^{-1}
TIRAP-rs561	rs657494561	29:28517376	[CTC/–]	0.151	3×10^{-5}	0.16	0.19	0.13	3.1×10^{-1}
PTPRT-rs588	rs667380588	13:69159682	[AAAACA/–]	1.000	0.022	0.13	0.13	0.13	1.0
KALRN-rs989	rs667660989	1:67361424	[TTGAA/–]	0.403	1.000	0.05	0.09	0.01	$\mathbf{5.3 \times 10^{-3}}$
RAB5a-rs016	rs661537016	1:154668023	[AACATATATA/–]	0.740	0.232	0.25	0.27	0.24	7.7×10^{-1}

Table 2
Genotype frequencies and association analysis. Significant and borderline p-values are indicated in black and grey bold, respectively.

SNP	Genotype	N° of:			Association study	
		Total	Control	Cases	OR (95% CI)	p-value
IRF3-rs540	AA	38 (0.30)	11 (0.17)	27 (0.42)	(Aa + aa) vs AA	**3.4×10^{-3}**
	Aa	70 (0.55)	38 (0.59)	32 (0.50)	3.52 (1.55–7.96)	
	aa	20 (0.15)	15 (0.24)	5 (0.08)		
FKBP5-rs294	Non-Ff	123 (0.96)	60 (0.94)	63 (0.98)	Ff vs non-Ff	3.7×10^{-1}
	Ff	5 (0.04)	4 (0.06)	1 (0.02)	4.20 (0.46–38.68)	
	ff	0 (0.00)	0 (0.00)	0 (0.00)		
TIRAP-rs561	TT	96 (0.75)	44 (0.69)	52 (0.81)	Tt vs (TT + tt)	6.4×10^{-2}
	Tt	23 (0.18)	16 (0.25)	7 (0.11)	2.70 (1.03–7.15)	
	tt	9 (0.07)	4 (0.06)	5 (0.08)		
PTPRT-rs588	RR	100 (0.78)	49 (0.76)	51 (0.80)	Pp vs (PP + pp)	5.0×10^{-1}
	Rr	24 (0.19)	14 (0.22)	10 (0.15)	1.51 (0.62–3.71)	
	rr	4 (0.03)	1 (0.02)	3 (0.05)		
KALRN-rs989	KK	117 (0.91)	54 (0.84)	63 (0.98)	(Kk + kk) vs KK	**8.6×10^{-3}**
	Kk	10 (0.08)	9 (0.14)	1 (0.02)	11.67 (1.45–94.14)	
	kk	1 (0.01)	1 (0.02)	0 (0.00)		
RAB5a-rs016	BB	69 (0.54)	34 (0.53)	35 (0.55)	bb vs (BB + bb) 2.01 (0.37–11.71)	6.8×10^{-1}
	Bb	53 (0.41)	26 (0.41)	27 (0.42)		
	bb	6 (0.05)	4 (0.06)	2 (0.03)		

KALRN-989 minor allele genotypes were almost exclusively present among controls (0.16 vs 0.02). Furthermore, a borderline significant association was detected at *KALRN*-989 (p = 0.008). Although the frequency of the *TIRAP*-561 heterozygous genotype was lower in cases (0.11) than in controls (0.25), this genotype was not significantly associated with absence of *Brucella*-specific antibodies (p = 0.06). To clarify the suggestive or significant associations, we performed *in silico* functional analysis and linkage disequilibrium (LD) tests with previously reported causal variants.

3.3. TIRAp-561

Although not significant, the *TIRAP*-561 association results that suggest a heterozygote advantage are in concordance with previous reports. Original studies showed that heterozygosity at two common SNPs in exon 5 of the human *TIRAP* gene (S180L and D96N) are associated with increased resistance to human tuberculosis and other infectious diseases [12,13]. The local alignment with BLAST showed a high degree of identity (79%) between caprine and human *TIRAP* exon 5 (data not shown). In an attempt to identify coding variants in linkage disequilibrium (LD) with InDel *TIRAP*-561, we performed direct sequencing of *TIRAP* exon 5 in 18 creole goats. While the homologous site of the human SNPs S180L and D96N were not polymorphic in the evaluated caprine population, two polymorphic variants (rs667345914 and rs655313459) were identified in exon 5 of the caprine *TIRAP* gene, but neither of these SNPs were in LD with *TIRAP*-561 (r^2 = 0.07 and 0.03, data not shown). Otherwise, *TIRAP*-561 was predicted to have no functional significance by *in silico* analysis with ESEfinder and RegRNA. Overall, our results do not support an association of *TIRAP*-561 with *Brucella*-specific antibodies status.

3.4. KALRn-989

It is known that alternative splicing of the human *KALRN* gene produces multiple isoforms with different functional domains [22]. Since *KALRN*-989 is located close to the 5′ end of exon 20, we hypothesized that it might affect *KALRN* pre-RNA splicing. Using ESE finder and RegRNA software, *KALRN*-989 was predicted to not alter splicing sites or splicing regulatory motives. Additionally, the *in silico* results showed that the minor allele (k) abolish putative binding sites for microRNAs mIR-138 and mIR-3065-3p. Although microRNAs binding sites located in intronic regions are not typically considered functional, some articles suggest that microRNAs may modulate constitutive and alternative splicing [23]. Otherwise, a non-synonymous SNP was previously identified at *KARLN* exon 20 in wild goats (rs684211905), but we did not observe any polymorphism within this exon in our domestic goat population.

3.5. IRf3-540

Upstream open reading frames (uORFs) are mRNA elements formed by a start codon (uAUG) within a 5′ UTR, and an in-frame stop codon (uSTOP) upstream or downstream of the main AUG (mAUG). These regulatory elements affect the translation of the main ORF by triggering mRNA decay or by blocking the translational machinery [24]. Using ESEfinder and UTRScan, we observed that the InDel *IRF3*-540 was located within an upstream open reading frame (uORF1) sequence, and that the minor allele “a” eliminated the uSTOP codon extending the uORF by 75 nucleotides (Fig. 1a and 2). The *IRF3* uORF1 had 3 of the 4 properties that are associated with functional strong uORFs: long distance between the 5’cap and the uAUG, strong uAUG context, and evolutionary conservation [25]. The *IRF3* uAUG1 was surrounded by a “strong” Kozak sequence and located approximately 93 nucleotides from the 5′ cap (Fig. 2). These two features are correlated with increased recognition of the uAUG by the ribosome [24].

The uORFs that have functional significance are likely to be retained among species [26]. Sequence alignment of the uORF1 region of the caprine *IRF3* gene with the equivalent 5′ UTR regions of cattle and sheep showed a high degree of identity (94% and 90%). Indeed, the uAUG1 context sequence was strongly conserved among goats, sheep and cows (Fig. 2). Comparative analysis of the 5′UTR structure with UTRScan (Fig. 1b) showed that the distance uAUG1-mAUG was conserved among goats (233 nt), sheep (230 nt) and cows (224 nt), and that the distance uSTOP1-mAUG and the uORF1 length were conserved between goats (96 nt and 138 nt) and sheep (96 nt and 135 nt). Further, a strong uORF was also detected in humans. Although the uORF1 was not conserved in sequence, the distance uSTOP-mAUG was similar between humans (106nt) and goats (96nt). Therefore, the position and coding content of the *IRF3* uORF1 are conserved among species, suggesting that these features may be important for the regulation of *IRF3* translation. In summary, this results shown that the deletion *IRF3*-540 (allele a) eliminated the STOP codon of a putative strong uORF,

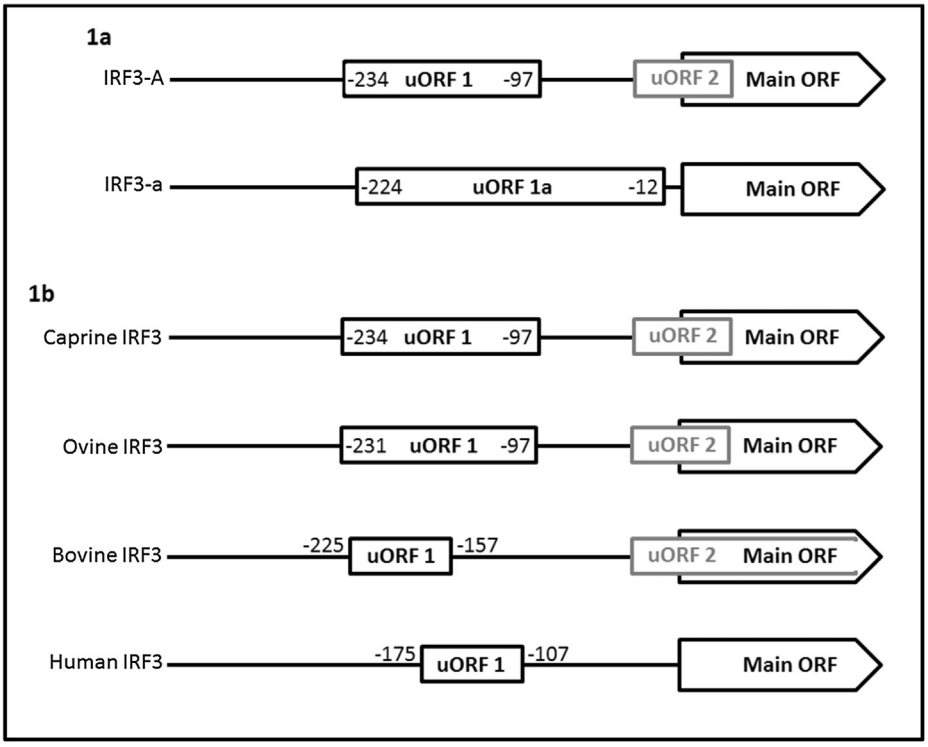

Fig. 1. Schematic representation of *IRF3* 5′UTR with upstream open reading frames (uORF). (A) Structure comparison between caprine *IRF3*-540 alelles. (B) Structure comparison between caprine, ovine, bovine and human *IRF3* 5′ UTR. The 5′ UTR structures were predicted by UTRScan software. The uORF2 start codon was surrounded by a "poor" Kozak sequence, thus the uORF2 was predicted to be non-functional or weak. Base positions for each uORF start and stop codons are given.

decreasing the conserved distance uSTOP1-mAUG from 96 nt to 11 nt. Because the a-allele uORF extends to the main ORF, the translation reinitiation at the main ORF could be reduced, leading to a stronger repression of *IRF3* expression.

3.6. The IRF3-540 a-allele is associated with resistance to Brucella infection in a dose-dependent manner

Further examination of *IRF3*-540 genotype frequencies suggests that the aa-genotype confers an increased resistance to *Brucella* infection with respect to the Aa-genotype. While the aa-genotype was present in a higher proportion of control than cases (0.28 vs 0.08), the frequency of the Aa-genotype was similar between groups (0.59 vs 0.50) (Table 2). To test this hypothesis, we reevaluated the genetic association of *IRF3*-540 using a logistic regression analysis under an additive model which assumes a linear relationship between the number of a-alleles and the resistance to *Brucella* infection [27]. The logistic regression model was statistically significant (p = 0.0004) and showed that each copy of the a-allele increased the protection against *Brucella* infection by about two-fold (p = 0.001; OR = 2.76; 95% CI = 2.52–5.00) with respect to the AA genotype.

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C. hircus 191 GAAAGGATGGGTGTGCATTGACAGTGGAGAAGGCGGGTTGTGTAATGTGGGGAAAGTGAA
O. aries  281 GAAAGGATGGGTGTGCATTGACAGTGGAGAAGGCGGGCTGTGTAATGTGGGGAAAGTGAA
B.taurus  273 GAAAGGATGGGTGTGCATTGACAGTGGGGAAGGCGG-----GTAATCTGGGGAAAGTGAA

C. hircus 251 ACAAGCAGGCAAAAAAGTGAAGCAATAGCAGGCGCCGAGGTCGGCTGTAAAACAAACAAA
O. aries  341 ACAAGCAGGGAAAAAAATGAAGCAATAGCAGTTGCCGAGGTCGGCTGTAAAACAAACAAA
B.taurus  328 ACAAGCAGGGAAAAAAGTGAAGCAATAGCAGACGCCCAGGTCGGCTGTAAAACAAACAAA

C. hircus 311 CAAA-AACAAACTCAG-------AAGGAGTAGCAAGGAGGAGGGGAGAGGAGGCATCGGT
O. aries  401 CAAA-AACAAACTCAGGAGTAGCAAGGAG----------GAGGGGAGAGGAGGCATCTGT
B.taurus  388 CAAACAAAAACAAACGGAGTTGCAAGGAG----------GAGGGGAGAGGAGGCATCTAT

C. hircus 353 ATTGGCGGATCTCGGGAGAGAAGGATCACTCTCCGAATGGGAGGCAGTCCACTCTAGAAA
O. aries  450 ATTGGCGGATCTCGGGAGAGAAGGATCACTCCGCGAATGGGAGGCAGTCCACTCTAGAAA
B.taurus  438 ATTGGCTGATCTCGGGAGAGAAGGATCACTG--CGAATGGGAGGCAGTCCACTCTAGAAA
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Fig. 2. Sequence comparison of the caprine, ovine and bovine *IRF3* uORF1 region. The figure shows the alignment of partial nucleotide sequences of the *IRF3* 5′ UTR of goat (*C. hircus*; XM_013971473.2), sheep (*O. aries*; XM_012157808.2) and cow (*B. taurus*; XM_015458163.1). Start and stop codons are indicated in bold. The uAUG1 strong Kozak sequence (A at −3 and G at +4 from the A at the uAUG) is highlighted in light grey and the InDel rs660531540 in dark grey. uORFs are defined by a start codon (**ATG**) in the 5′ UTR, an in-frame stop codon (**TAG**, **TGA**) and length ≥9 nt.

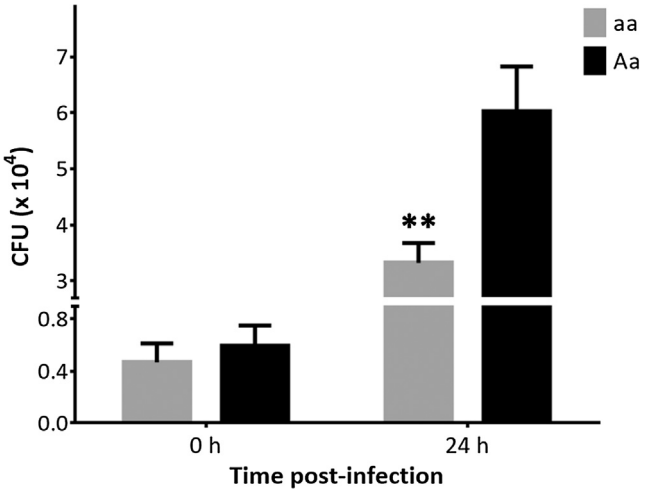

Fig. 3. Intracellular bacteria recovered from aa- or Aa-MDMs at 0 and 24 h post-infection with *B. melitensis* 16M. Bacteria were counted by the CFU method. The experiment included seven Aa- and seven aa-goats, each tested twice in independent experiments. Data were represented as the means ± standard error of the mean (SEM); **p ≤ 0.01.

3.7. The aa-MDMs present a B. melitensis intracellular growth restrictive phenotype

To evaluate the biological relevance of *IRF3*-540 InDel in host resistance to *B. melitensis* infection, we proposed to study the effect of *IRF3*-540 genotypes on the invasion and intracellular persistence of *B. melitensis* in caprine macrophages. The absence of *IRF*-540AA genotype among a group of goats randomly analyzed restricted our *in vitro* study to the other two genotypes. MDMs from seven aa and seven Aa goats were infected *in vitro* with *B. melitensis* 16 M and the number of intracellular bacteria was determined by a gentamicin-protection assay (Fig. 3). At an early time of infection (T0), no significant differences were observed in the recovery of intracellular bacteria between aa- and Aa-MDMs, suggesting that the *IRF3*-540 polymorphism does not affect the attachment and internalization of *B. melitensis* in macrophages. However, at 24 h post-infection, aa-MDMs had a reduced number of intracellular *Brucella* compared with the Aa-MDMs ($p \leq 0.01$), indicating that the aa-MDMs control more efficiently the intracellular grown of *B. melitensis* than the Aa-MDMs.

Altogether, the results show here support the association of the *IRF3*-540 a-allele with resistance to *B. melitensis* infection.

4. Discussion

Increasing evidence indicates that host genetic factors influence the outcome of exposure to *Brucella* spp. [5,10,18], but the underlined mechanisms that influence pathogen resistance are poorly known. Thereby, one of the main aims of candidate gene association studies is the identification of functional variants, which is a crucial step for understanding the molecular basis of genetic resistance to bacterial diseases. Here, we show a study of the relationship of six InDel alleles and genotypes with absence/presence of *Brucella*-specific antibodies in Argentinian creole cross-breed goats. It is widely accepted that the detection of *Brucella*-specific antibodies by accurate diagnosis tests is a strong indicator of *Brucella* infection in unvaccinated adult animals, and that resistant animals, when exposed to *Brucella*, develop only low transient serologic titers [28]. Therefore, serology test results are commonly used to define the resistance-susceptibility phenotypes in genetic association studies for infection with *Brucella* ssp. and other facultative intracellular bacteria [11,18,29]. The candidate gene analysis reported here identified significant associations between resistance to *Brucella* spp. infection and variants at *KALRN* (at allelic level) and *IRF3* (at both allelic and genotypic level). Furthermore, our *in silico* and *in vitro* analyses support a functional role of *IRF3* InDel rs660531540 on the regulation of *IRF3* translation and on the ability of macrophages to control the intracellular grown of *B. melitensis*, respectively.

Interferons (IFNs) are important cytokines in the immune responses to intracellular pathogens and tumor cells [30,31]. Classically, type I IFNs (IFN-α, β, ω, κ and ε) are associated with antiviral responses, whereas IFNγ enhances antibacterial immunity. However, a growing appreciation that the IFN-α/β plays also an important role in the host response to bacterial infection arise lately, despite of elevated IFN-α/β levels has been associated with disease progression in chronic infections [30,32]. Activation of type I IFNs by bacterial DNA mostly requires IRF3 and is MyD88-dependent [7,33]. IRF3 is a member of the interferon regulatory transcription factor (IRF) family that plays a critical role in both the early and late phases of the IFN-α/β gene induction. Upon infection, phosphorylated IRF3 translocates to the nucleus and activates the transcription of type I IFN genes and IFN-stimulated genes by binding to an interferon-stimulated response element in their promoters [34]. The implication of *IRF3* during *Brucella abortus* infection was previously described in a mouse model, which showed that *B. abortus* induces IFN-β expression dependent of IRF3 but independent of Toll-like receptors (TLRs) [6]. Indeed, that study also reported that mice lacking the IFN-α/β receptor are more resistant to infection suggesting that IFN-α/β is detrimental to host control of *B. abortus*. More recently, a computational analysis predicted a putative protein-protein interaction between *Brucella* BMEI1751 (luxR family) and bovine *IRF3* at the onset of the *B. melitensis* infection process [35].

Previously, another research group identified a SNP in intron 5 of the murine *IRF3* gene that disrupts a U12-type branch point and has a negative effect on the efficiency of *IRF3* splicing. This polymorphism reduces the IRF3 protein levels and confers protection to *Listeria monocytogenes* infection in mice [8]. Here, we reported that the *IRF3* InDel rs660531540 (*IRF3*-540) disrupts a conserved uORF sequence (Figs. 1 and 2), and would be strongly associated with increased resistance to *Brucella* spp. infection in goats based on serology results (Tables 1 and 2). Eukaryotic ribosomes usually load on the 5′ cap of mRNA transcripts, scan downstream, and initiates translation at the first AUG start codon with a favorable sequence context, thus uORFs can reduce the efficient translation of the downstream coding sequence [25,26]. Ribosomes encountering a uAUG can translate the uORF and then, with some probability, reinitiate to translate the main ORF. Previous reports suggested that re-initiation efficiency is inversely related to the uORF length and directly related to the intercistronic distance (uSTOP-main AUG) [24,36]. Since *IRF3* uORF is conserved between species and the *IRF3*-540 deletion (minor allele) decrease the intercistronic distance and extend de uORF length, it is possible that this deletion alters the regulation of *IRF3* translation during *Brucella* infection in goats. As far as we know there are no previous reports describing uORF motifs in *IRF3* genes, and little is known about the mechanism that up-regulates *IRF3* expression during pathogen infection. It was reported that a mutation in another IRF family member (*IRF6*) creates a uORF that decreases translation efficiency from the main ORF, and causes Van der Woude syndrome [25]. The present work encourages future *in vitro* studies about the functional importance of the *IRF3* uORF, and the effect of the InDel *IRF3*-540 on IRF3 translation and *Brucella* pathogenesis.

The *KARLN* InDel rs667660989 minor allele was also significantly associated with resistance to caprine brucellosis (Table 1), but this association was not significant at genotypic level (Table 2). Previously, a genome-wide association study (GWAS) in dairy cattle detected a strong association between *KARLN* SNP BovineHD0100019801 and resistance to infection with *Mycobacterium bovis*, another facultative intracellular pathogen [11]. Although *KARLN* has an unknown function in immune response, these association studies provide support for further investigations about the function of *KARLN* in immune cells and its role in host resistance to infections.

This study did not detect significant associations at InDels *FKBP5*-294, *TIRAP*-561, *PTPRT*-588 and *RAB5a*-016. Only studies with very large sample sizes are adequately powered to detect low frequency variants that exert a small effect [37]. Thereby, these lacks of association could be related to the small sample size utilized, particularly at *TIRAP*-561 whose genotype frequencies were apparently different between control and cases (Table 2), and were severely out of Hardy Weinberg equilibrium among cases (Table 1). It is accepted that deviation from Hardy-Weinberg proportions in cases may indicate association when it holds in controls [20].

It would be useful to deep this study evaluating a higher number of animals to validate these results. In conclusion, this is the first study showing a genetic association between *IRF3* variants and absence of brucellosis in a higher mammal. Altogether, the results reported here contribute to the identification of the genetic mechanisms that influence the resistance against intracellular bacterial pathogens.

Acknowledgement

The authors want to thank to Dr. Ema M. Cano Pereira for experimental advice. We also thank Dr. Cristina Rossetto and Mr. Antonio Salatin for helping us during farm work. This study was supported by the Agencia Nacional de Promoción Científica y Tecnológica de Argentina (Grant PICT 2014-866) and INTA (Grant PNBIO 1131033).

Conflict of interest

None.

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