

ORIGINAL ARTICLE

Novel variants within the coding regions of the *Slc11A1* gene identified in *Bos taurus* and *Bos indicus* breedsR. Martínez¹, S. Dunner², G. Barrera¹ & J. Cañon²

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Although in the cow the genetic resistance to brucellosis has been previously attributed to the *Slc11A1* gene encoding Nramp1 protein, none of the mutations described to date seems to be the cause. To be able to associate another polymorphism of the gene to brucellosis resistance, we characterized the gene and identified in different breeds of *Bos taurus* and *Bos indicus*, six new variants among a total of 11 single nucleotide mutations, of which five occurred in the coding sequence (three are missense mutations), one in the promoter region and five in introns. The allelic and genotypic frequencies calculated revealed differences ($p < 0.05$) among the breeds studied.

Breeding domestic animals for genetic resistance to brucellosis or salmonellosis is an attractive alternative to the use of control methods for reducing morbidity. The *natural resistance associated macrophage protein-1* (NRAMP 1) gene, formally called Solute carrier family 11 member 1 gene (*Slc11A1*), has been associated with susceptibility/resistance to many intracellular pathogens and encodes a divalent cation transporter located in the phagolysosome membrane of macrophages. It has been shown to play a critical role in innate immunity, promoting bacterial killing by macrophages in addition to its effects on adaptive immunity in mice (Vidal et al. 1995). In cattle, this gene has been associated with resistance against *Brucella abortus* infection (Feng et al. 1996; Adams and Templeton 1998; Horin et al. 1999; Barthel et al. 2001), although there are contradictory findings, as Kumar et al. (2005), and Paixao et al. (2007) did not detect association between a 3'UTR polymorphism and resistance to brucellosis in cattle. Interestingly, Borriello et al. (2006) and Capparelli et al. (2006) studying the two Nramp1 alleles located in the 3'UTR of the water buffalo Nramp1 gene, found dif-

ferences in the number of GT repeats (33 in the A allele and 36 in B) and detected that the Nramp1AA genotype confers susceptibility to *Brucella abortus* in water buffalo.

Further, characterizing this gene is an interesting enterprise due to its importance in brucellosis reported by several authors. Here, we identify new variants in the coding regions of the *Slc11A1* gene as a necessary step in increasing the knowledge about the gene.

To amplify the *Slc11A1* gene, primers were designed based on GenBank accession number AC149748 and on information found by Coussens et al. (2004) (Table S1). Fourteen bovine individuals [10 *Bos taurus* specifically Colombian Creole Blancorejinegro (BON) and four Zebu (*Bos indicus*)] previously evaluated for brucellosis resistance (Martínez et al. 2005) by *in vitro* macrophage infection [following the methodology reported by Templeton et al. (1990) and Qureshi et al. (1996)] were selected and blood sampled. The DNA samples were subjected to exon amplifications using *Taq* DNA Polymerase (Bio-tools, Madrid, Spain) through an initial incubation

at 94°C for 5 min, followed by 33 cycles of 95°C for 1 min, 56°C for 1 min and 72°C for 1 min and ended by 4 min at 72°C. To detect mutations, 20–100 ng of the amplicons were directly [or after digestion with restriction enzymes depending on their size (Table S1)] subjected to single strand conformation polymorphism (SSCP) analysis by mixing the amplicons (v/v) with denaturing 2x SSCP loading buffer (95% formamide, 0.6% bromophenol blue and 0.6% xylene cyanole), by heating at 94°C for 5 min, snap chilling on ice and loading onto 29:1 acrylamide:bis-acrylamide 8% polyacrylamide gels prepared with TBE (Tris, Boric Acid, EDTA) buffer. Electrophoresis was allowed to run at 10°C and 4 W for 15 h and gels were then silver-stained (described in Bassam et al. 1991 modified by Barroso et al. 1997). The polymorphic PCR fragments were purified (Roche Diagnostics, Mannheim, Germany) and directly sequenced with a Big Dye sequencing kit (Applied Biosystems, Darmstadt, Germany) in an ABI 3130 automated DNA sequencer using both primers. Sequences were edited using ClustalW 1.82 software (Thompson et al. 1994) and compared with GenBank accession number U12862. Seventy-two unrelated animals from seven different cattle breeds were subsequently genotyped using SSCP analysis: Zebu ($n = 11$); two Colombian breeds: BON ($n = 11$) and Romosinuano (ROMO; $n = 10$); Holstein (H; $n = 10$) and three Spanish beef breeds: Asturiana de los Valles (AV; $n = 10$), Rubia Gallega (RG; $n = 10$) and Pirenaica (PI; $n = 10$) for six of the identified mutations [those located in promoter and coding regions: SNP1_nt151 (promoter c.-93C > T); SNP2_aa29 (g.870A > G); SNP3_aa272 (p.A272V); SNP4_aa321 (p.D321N); SNP5_aa356 (p.P356A); SNP6_aa542 (p.Q542del)], and allelic and haplotype frequencies were computed by breed using ARLEQUIN Software 3.01 (Excoffier et al. 2005).

The newly described full sequences for *Slc11A1* were submitted to GenBank under accession numbers DQ493965 and DQ493966.

Eleven informative bands in nine regions of the gene sequenced for a group of 14 BON (*Bos taurus*) and Zebu breed (*Bos indicus*) animals yielded 10.665 bp and one single nucleotide polymorphism (SNP) at every 969 bp on average, which is lower than in cytokine genes (one SNP per 75 to 237 bp (Heaton et al. 2001) and points to a high-selection pressure acting on this gene. In total, eight transitions, two transversions and one deletion were observed (Figure 1), with their eventual amino acid change being described in Table S1. Three of the newly described polymorphisms cause a change at p.A272V in exon 9, at p.D321N in exon 10 and at p.P356A in exon 11. A three nucleotide deletion at position 542 causes a glutamine deletion (p.Q542del) in exon terminal 15. Some of the non-coding polymorphisms were newly detected (g.2622G > A, g.2647C > T, g.3582C > T), but we did not find others as is the case for deletion in intron 6 (AY840992) and a three 'G' insertions in intron 10 (Coussens et al. 2004) despite the fact that the latter is described as widely distributed (showing a frequency of 0.65 and 0.35 for wild type and mutant alleles respectively) within *Bos taurus* populations. Also, the g.1202C > T located in exon 5, (Ile to Thr) was not detected in this study (Ables et al. 2002). All sequence variations are showed in Table S1. Based on the degree of conservation among species, a change from glycine at amino acid position 169 in exon 7 to asparagine might have been expected in the disease-sensitive genotype (Malo and Skamene 1994) as it occurs in the murine species. However, all animals examined in our study had the glycine residue, as reported by Feng et al. (1996).

The secondary structure predicted with NNPREDICT software (Kneller et al. 1990) revealed changes in exons 9 and 11. The first appeared between transmembrane domains 6 and 7 and produced a strand (E) at a site with no predicted secondary structure, modifying the resultant helix structure. Further changes were produced in exon 11 within trans-

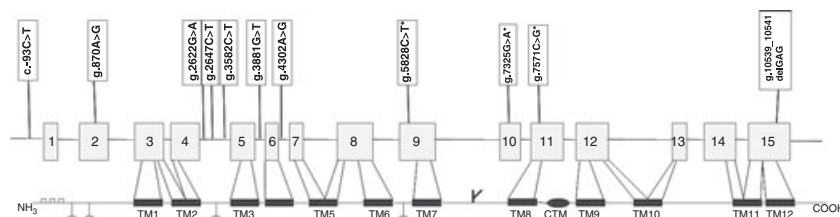


Figure 1 Genomic structure and polymorphisms of the *Slc11A1* gene (boxes indicate exons and lines connecting them indicate introns). single nucleotide polymorphisms (SNPs) are indicated according to their position in sequence DQ493965 and protein structure (TM, trans-membrane domain; CTM, consensus transport motif).

Bos taurus	MSGDTGPPKQGGTRYGSISSPPS---PEPQQAPPGGTYLSEKIPIDTESGTFSLRKLWA	57
Bos indicus	MSGDTGPPKQGGTRYGSISSPPS---PEPQQAPPGGTYLSEKIPIDTESGTFSLRKLWA	57
Ovis aries	MSGDTGTPNQGGTRYGSISSPPS---PGPQQAPPGGTYLSEKIPIDTESGAFSLRKLWA	57
Homo sapiens	MTGDKGPPQLSGSSYGSISSPTSPTSPGPQQAPPRETYLSEKIPIDTKPGTFSLRKLWA	60
Mus musculus	MISDKSPRLSRPSYGSISSLPG---PAPQPAPCRETYLSEKIPIPSDAQGTFSLRKLWA	57
Bos taurus	LLQAVGIIGAIIMPHNIYLHSSLVKSREVDRSRRADIREANMYFLIEATIALSVSFLINL	297
Bos indicus	LLQAVGIIGAIIMPHNIYLHSSLVKSREVDRSRRADIREANMYFLIEATIALSVSFLINL	297
Ovis aries	LLQAVGIVGAIIMPHNIYLHSSLVKSREVDRSRRADIREANMYFLIEATIALSVSFFINL	297
Homo sapiens	LLQAVGIVGAIIMPHNIYLHSLVKSREIDRARRADIREANMYFLIEATIALSVSFFINL	300
Mus musculus	LLQAVGIVGAIIMPHNIYLHSLVKSREVDRTRRVDVREANMYFLIEATIALSVSFFINL	297
Bos taurus	FVMAVFGQAFYKQTNQAAFNICADSSLHDYAPIFPRNLTVAVDIYQGGVILGCLFGPAA	357
Bos indicus	FVMAVFGQAFYKQTNQAAFNICANSSLHDYAPIFPRNLTVAVDIYQGGVILGCLFGPAA	357
Ovis aries	FVMAVFGQAFYKQTNQAAFNICANSSLHDYATIFPRNLTVAVDIYQGGVILGCLFGPAA	357
Homo sapiens	FVMAVFGQAFYKQTNQAAFNICANSSLHDYAKIFPMNNTVAVDIYQGGVILGCLFGPAA	360
Mus musculus	FVMAVFGQAFYQQTNEAFNICANSSLQNYAKIFPRDNNTVSDVIYQGGVILGCLFGPAA	357
Bos taurus	EEDQEKGRITSG	548
Bos indicus	EEDQ-KGRITSG	547
Ovis aries	GEDQEEGRITSG	548
Homo sapiens	EEDQ-KGETSG	550
Mus musculus	NEEQGGVQGS	548

Figure 2 Amino acid variations in the Nrap1 protein encoded by the *Slc11A1* gene for several species.

membrane domain 8, generating a downstream variation that gives rise to a strand instead of a helix structure.

Alignment using ClustalW (Thompson et al. 1994) with other species (Figure 2), murine (NP_038640), human (NP000569), ovine (AAC28241), suggests that mutations at the conserved residues at amino acid positions 321, 356, and deletion at 542 can generate significant trans-membrane domain changes. These variations can be a better candidates to explain the different degrees of brucellosis resistance observed in the different breeds, following the contradictory findings in the 3' UTR effect on susceptibility in cattle (Paixao et al. 2007). The finding is to be confirmed in the near future by searching for a possible association between these mutations and resistance measured through macrophage *in vitro* killing assays.

Genotypic and allelic frequencies were calculated for 72 unrelated individuals belonging to seven different breeds (Table 1) as a first step. This small sample was used to assess frequency status of the six more important mutations in European milk and meat breeds. The occurrence of different alleles and genotypes varies significantly among breeds ($p < 0.05$). Only SNP 2 and SNP 5 showed a homozygous mutated genotype with a frequency >0.2 . SNP3 located at exon 9 showed low variability among breeds, with only Zebu and BON emerging as breeds in which the wild type allele was not fixed. The SNP4_GG allele is not present in Zebu, where SNP4_AA is fixed, the latter also appears in the *Bos taurus* BON breed.

Heterozygous genotypes were found at null or low frequencies except for SNP5 in exon 11 in the local Spanish and ROMO breeds, and for SNP6 in the Zebu and Rubia Gallega breeds. SNP4 exhibited no

heterozygotes in any breed. The BON breed rendered interesting frequencies between 0.18 and 0.27 for the first three SNPs.

All 27 haplotypes described showed an overall frequency of <0.10 except for haplotype 1 and 2. Table 1 shows the results for the four most frequent haplotypes. It should be noted that all the *Bos taurus* breeds display the same common haplotypes, with frequencies above 0.2. Haplotype 1 appeared as fixed and unique in Holstein and with similar frequencies in ROMO, BON and AV. This high frequency in Holstein breed could be the consequence of a selective pressure, while in the other breeds the results could be explained by random drift due to low effective population size (Gutierrez et al. 2003). The most common haplotype present in Zebu individuals had the exon 15 deletion. In general, the Zebu breed showed several haplotypes that were not found in other breeds since some mutations only occurred in the Zebu breed. For the 3'UTR *Slc11A1* polymorphism, a marked difference in allele frequencies was also detected between the Zebu and Holstein cattle (Paixao et al. 2006); Holsteins had an extremely homogeneous genotype with 100% of the individuals bearing the GT₁₃ genotype.

In conclusion, we observed moderate variation in the coding sequence of the *Slc11A1* gene in cattle. This could be a sign of selection pressure on this key gene, which has previously been shown to bear a 3'UTR polymorphism. As the link of the latter polymorphism with brucellosis resistance in *Bos indicus* and *Bos taurus* \times *Bos indicus* populations was not demonstrated by Kumar et al. (2005), the systematic characterization of the *Slc11A1* gene in terms of new polymorphisms in different breeds showing resistance or susceptibility to brucellosis is important. It will facilitate the way to identify the mechanisms

Table 1 Genotype frequencies for 6 SNPs of the *Slc11A1* gene in 72 animals of seven *Bos taurus* and *Bos indicus* breeds (10 or 11 per breed) and description of the four most frequent haplotypes and their frequencies within and among breeds

Genotype	SNP1_nt151 (5'UTR)						SNP2_aa29			SNP3_aa272			SNP4_aa321			SNP5_aa356			SNP6_aa542			Haplotypes frequencies							
	C	C/T	T	A	A/G	G	C	C/T	T	G	A	C	C/G	G	GAG	GAG	del/	GAG/	del/	GAG	G	GAG	del/	del/	(Haplo 1) CACGCTCAG GAGAAG	(Haplo 2) CACGGTCAG GAGAAG	(Haplo 3) CGCGCTCA GGAGAAG	(Haplo 4) TGCAGTCAG ---AAG	
BON (n = 11)	0.73	0.18	0.09	0.82	0.18	0.00	0.73	0.27	0.00	0.73	0.27	0.64	0.00	0.36	1.00	0.00	0.00	0.00	0.00	0.36	1.00	0.00	0.00	0.54 ± 0.16					
ZEBU (n = 11)	0.00	0.09	0.91	0.09	0.09	0.82	0.64	0.00	0.36	0.00	1.00	0.18	0.09	0.73	0.27	0.27	0.45										0.27 ± 0.14		
RG (n = 10)	1.00	0.00	0.00	0.90	0.00	0.10	1.00	0.00	0.00	1.00	0.00	0.30	0.50	0.20	0.30	0.40	0.30	0.30	0.30	0.20	0.20	0.13	0.20	0.20 ± 0.13	0.20 ± 0.13				
AV (n = 10)	0.90	0.00	0.10	0.70	0.00	0.30	0.90	0.00	0.10	1.00	0.00	0.80	0.20	0.00	1.00	0.00	0.00	0.00	0.00	0.40	0.40	0.16							
PI (n = 10)	1.00	0.00	0.00	0.40	0.00	0.60	1.00	0.00	0.00	1.00	0.00	0.30	0.50	0.20	0.70	0.30	0.00	0.30	0.00	0.20	0.20	0.13	0.20	0.20 ± 0.13	0.20 ± 0.13				
H (n = 10)	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	1.00 ± 0.00					
ROMO (n = 10)	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.50	0.50	0.00	1.00	0.00	0.00	0.00	0.50	0.50	0.16	0.50	0.50 ± 0.16	0.50 ± 0.16					
Freq ±	0.78 ±	0.04 ±	0.17 ±	0.69 ±	0.04 ±	0.26 ±	0.89 ±	0.04 ±	0.07 ±	0.82 ±	0.18 ±	0.22 ±	0.25 ±	0.52 ±	0.75 ±	0.14 ±	0.11 ±	0.39 ±	0.06	0.12 ±	0.04	0.04 ±	0.02	0.03 ±	0.02	0.03 ±	0.02		
SD	0.05	0.02	0.04	0.05	0.02	0.05	0.04	0.02	0.03	0.04	0.04	0.05	0.05	0.06	0.05	0.04	0.03	0.04	0.03	0.05	0.05	0.04	0.03	0.03	0.04	0.04	0.02		
Allelic freq	0.802	0.188	0.715	0.288	0.909	0.090	0.819	0.180	0.652	0.347	0.819	0.180	0.819	0.180	0.819	0.180	0.180	0.180	0.180	0.180	0.180	0.180	0.180	0.180	0.180	0.180	0.180	0.180	0.180

BON, Blancorejnego; ZEBU, zebu; RG, Rubia Gallega; AV, Asturiana de Valles; PI, Pirenaica; H, Holstein; ROMO, Romosinuano.

through which a *Bos taurus* or *Bos indicus* animal can show resistance towards a *Brucella* infection. We have worked under way to associate the presence of these particular alleles with individual animals showing resistance to brucellosis by measuring the ability of their macrophages to control bacterial replication *in vitro*.

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Supplementary material

The following supplementary material is available as part of the online article from <http://blackwell-synergy.com>:

Table S1 Primers and sequences used for PCR amplification of the *Slc11A1* gene coding region based on Genbank AC149748 and information on intron-exon junctions described by Coussens *et al.*

(2004). For each amplicon produced, digestion with restriction enzymes is indicated when used, and also the SNP identified and eventually the aa (amino acid) change (nt = nucleotide). Last column indicates other polymorphisms described by other authors (indicating author and GenBank accession number).

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