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Relationship of Bovine *SLC11A1* (Formerly *NRAMP1*) Polymorphisms to the Risk of Bovine Tuberculosis in Holstein Cattle

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Abstract

Many studies suggest significant genetic variation in the resistance of cattle and humans to infection with *Mycobacterium bovis*, the causative agent of zoonotic tuberculosis. The natural resistance associated macrophage protein 1 (NRAMP1), encoded by the *SLC11A1* gene, plays a key role in the immunological control of a broad spectrum of infectious agents. The aim of the present study was to investigate the influence of single nucleotide polymorphisms (SNPs) of the *SLC11A1* gene on bovine tuberculosis (bTB) susceptibility. We genotyped the *SLC11A1* gene in 60 bTB-infected Holstein cows and 90 healthy control animals. The influence in the exon 4 and intron 4 regions of *SLC11A1* genetic variations on bTB susceptibility was subsequently investigated by association analysis. Our finding demonstrated that the g.107117166A>G and g.107117369C>T polymorphisms of the *SLC11A1* gene associated with bTB in Holstein cattle. The susceptibility of cattle with the g.107117166A>G genotype compared with the GG genotype was 3.40 (95% CI: 1.10-10.51; p=0.048) fold higher. The g. 107117166A>G SNP located in the exon 4 of the *SLC11A1* gene and the functional consequence was missense. The deduced amino acid sequence for the protein product revealed an alanine to threonine conversion at position 96, which may affect initiation of protein synthesis and disrupt normal NRAMP1 function that protects animals against mycobacterial infection. The other susceptibility of cattle with the g.107117369C>T genotype compared with the TT genotype was 0.26 (95% CI: 0.12–0.56; p=0.001) fold lower. The g.107117369C>T polymorphism, located in the intron 4 of the *SLC11A1* gene, may affect elements that control transcription and splicing of the NRAMP1 leading to affect pathophysiological characteristics in tuberculosis. This is the first report showing that the g. 107117166A>G and g.107117369C>T polymorphisms may contribute to *SLC11A1*-mediated bTB susceptibility.

Keywords: Bovine tuberculosis; *SLC11A1*; Susceptibility

Introduction

Bovine tuberculosis (bTB) is caused by the intracellular pathogen *Mycobacterium bovis (M. bovis*), a facultative intracellular parasite of macrophages. This zoonotic infection has a significant economic impact and serious implications for human health, especially in developing countries [1]. Most bTB control programs rely on the intradermal tuberculin skin test (TST) to identify infected cattle, which are then culled [2]. In Taiwan, a national bTB eradication program–comprising an annual TST, restriction of animal movement and slaughter of reactor animals–has been implemented since 1947. Despite these efforts, tuberculosis in animals and humans is still present, and the proportion of TST-positive cattle increased from 0.22% in 2000 to 0.36% in 2002 [3]. The failure of the present measures to eradicate bTB necessitates consideration of additional or complementary control measures.

M. bovis can be transmitted to humans via infectious bacilli through respiratory contact with infected cattle or consumption of unpasteurized dairy products [4]. The host immune response to *M. bovis* infection is complex; following initial exposure, the bacilli are phagocytosed by host macrophages via transporters such as natural resistance-associated macrophage protein 1 (NRAMP1), encoded by the solute carrier family 11 member 1 (*SLC11A1*) gene. This transporter – which functions as part of the innate immune response – plays a key role in inhibiting proliferation of *Mycobacterium tuberculosis (M. tuberculosis),* through its involvement in acidification of phagosomes [5]. The relationships between various polymorphisms in the human *SLC11A1* gene and mycobacterial diseases have been explored, including INT4 (single nucleotide G>C change in intron 4, 469+14G/C, rs3731865), D543N (conservative single base G>A substitution at codon 543, resulting in a change to asparagine from aspartic acid), and 3'UTR TGTG (a deletion in the 3' untranslated region, 1729+55del) [6-10]. Additionally, a Taiwanese aboriginal case-control study revealed that individuals possessing one of the three polymorphism of the *SLC11A1* gene have higher susceptibility to tuberculosis, with those who are heterozygous for the INT4 polymorphism being at greatest risk [6]. Similar findings have also been reported in Chinese, Korean, Japanese, Iranian, African-American and Caucasian populations [7-12]. In bovine studies, a genomic region of the 3'UTR of the *SLC11A1* cDNA gene sequence is associated with natural resistance against bTB. Polymorphisms in this region (alleles 211, 215 and 217) were associated with a significantly lower incidence of bTB in Zebu (*Bos indicus*) cattle [13-15]. However, Barthel et al. analyzed 33 cattle (breed unknown) with positive TST results, no association was found between resistance or susceptibility to infection with *M. bovis* and the 3'UTR microsatellite of the *SLC11A1* gene [16]. Moreover, recent findings have shown different levels of susceptibility to bTB among various cattle breeds [17-21].

Ameni et al. demonstrated high prevalence and increased severity of bTB pathology in Holstein (*Bos taurus*) compared with Zebu breeds; the risk of bTB in the Holstein breed was more than twice that of Zebu [17]. However, little information is available regarding the association between bTB susceptibility and single nucleotide polymorphisms (SNPs) in the exon 4 and intron 4 regions of the *SLC11A1* gene in Holstein cattle. Thus, the aim of the present study was to identify possible associations between SNPs of *SLC11A1* and the risk of bTB in Holstein cattle.

Materials and Methods

Animal studies

Our cohort comprised 150 Holstein cattle in Taiwan, (age range: 0.7–5.0 years), 60 of which were mandatorily sent to be culled from January of 2011 to December of 2014 after a positive TST, in line with regulations in Taiwan. The remaining 90 control animals, which were frequency-matched to the bTB population in terms of age and gender, were selected from a herd without a recent history of tuberculosis and were TST-negative. All procedures described in this study were reviewed and approved by the National Taiwan University Institutional Animal Care and Use Committee. Subjects with an *M. bovis* positive lymph node were confirmed bTB by culture. Associations between genetic polymorphisms and bTB were examined using a 1:1.5 matched case–control study.

Preparation of bovine genomic DNA

For DNA extraction, 5–10 ml of peripheral blood was collected from each animal, stored at −20°C, taken to the laboratory on dry ice. DNA from the blood samples was extracted using DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD, USA), according to the manufacturer's instructions. Concentration and purity of the extracted DNA was verified optically using an ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, DL, USA).

Genotyping by polymerase chain reaction

Polymerase chain reaction (PCR) and direct sequencing analysis were used to detect polymorphisms. Samples for PCR were prepared in a volume of 30 µl, consisting of 20 µl of diethyl pyrocarbonate-treated water, 3 µl of 10×PCR buffer, 0.5 µl of each primer (10 µM), 2 µl of 10 mM deoxynucleotide triphosphate (dNTP; Viogene BioTek Corp., New Taipei City, Taiwan), 0.5 µl of 2 units/µl Taq DNA polymerase (Viogene BioTek Corp.), and 4 µl of the extracted DNA. The forward 5′-TCT CTG GCT GAA GGC CTC TCC-3′ and reverse 5′- TGT GCT ATC AGT TTG AGC CTC-3′ primers were used for amplifying *SLC11A1* fragments, as described previously [21]. The reaction was initiated by heating the mixture to 94°C for 5 min, followed by 35 cycles at 94°C for 50 s, 55°C for 1 min, and 72°C for 1 min, after which the reaction was concluded with a final extension step at 72°C for 10 min. The PCR products were analyzed using 3% agarose gel electrophoresis. Based on a predicted size of 400 base pairs, products were then sequenced using a BigDye terminator cycle sequencing kit in an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Waltham, MA, USA), using the above-mentioned PCR primers.

Statistical methods

The chi-square test was used to examine the association between genetic polymorphisms and animal's susceptibility to infection. Statistical analyses were performed to compare allelic and genotypic distributions (which were analyzed using R Data Analysis & Guiding System, http://rweb.tmu.edu.tw/index.php). If the theoretical value for a cell was <5 in the chi-square test, Fisher's exact test was applied. The analyses were performed for all the polymorphisms of the gene assessed in this study. For each polymorphism, allelic analyses were performed to determine if animals had a homozygous or heterozygous genotype. To explore possible association between allele or genotype frequencies and infection status of cows, heterogeneity of odds ratios (ORs) for susceptibility to bTB infection was assessed. ORs and 95% confidence intervals (CIs) were calculated in order to estimate animals' risk of disease. Statistical significance was set at 5% (p<0.05).

Results

Polymorphisms in exon 4 and intron 4 of the bovine *SLC11A1* gene

The SNPs including g.107117166A>G, rs458688926, rs449522039 and g.107117369C>T were identified in exon 4 and intron 4 of the bovine *SLC11A1* gene. In order to detect the influence of *SLC11A1*-mediated bTB susceptibility, the following polymorphisms were detected: rs438480308, g.107117166A>G, rs458688926, rs478409125, rs440590557, rs460647173, rs717758716, rs480706808, rs449522039, rs469205385, g.107117369C>T, rs520905988, rs482869550, rs445253274, rs465479809, rs433744578, rs453907967, and rs524557355. In exon 4, the polymorphisms g.107117166A>G and rs458688926 (g.107117167G>C) were identified in both bTB-positive and control cattle. In intron 4, the rs449522039 (g.107117326T>A) and g.107117369C>T SNPs were identified. The influence of genetic variants on bTB susceptibility is presented in Table 1.

Effects of polymorphisms on bTB risk

The polymorphisms g.107117166A>G and g.107117369C>T of the *SLC11A1* gene were significantly associated with bTB infection in Holstein cattle. The susceptibility of cattle with the g.107117166A>G genotype compared with the GG genotype was 3.40 (95% CI: 1.10-10.51; p=0.048) fold higher. Furthermore, the A allele was a risk factor for predisposition to bTB (OR: 3.18; 95% CI: 1.06–9.56; p=0.055). The susceptibility of cattle with the g.107117369C>T genotype compared with the TT genotype was 0.26 (95% CI: 0.12–0.56; p=0.001) fold lower. There was a strong association between the C allele and lower risk of bTB in cattle (OR: 0.56; 95% CI: 0.34–0.92; p=0.030). Our associations remain significant after accounting for multiple testing (p=0.008). The results demonstrated the implications of g.107117166A>G and g.107117369C>T polymorphisms of the *SLC11A1* gene on bTB susceptibility in Holstein cattle.

The polymorphisms rs458688926 and rs449522039 of the *SLC11A1* gene were not significantly associated with bTB infection in Holstein cattle. The susceptibility of cattle with the rs458688926 genotype compared with the CC genotype was 2.84 (95% CI: 95% CI: 0.79–10.16; p=0.12) fold higher. The susceptibility of cattle with the rs449522039 genotype compared with the AA genotype was 3.07 (95% CI: 0.27–34.62; p=0.56) fold higher. The genotype and allelic distributions of infected and control animals are presented in Tables 2

and 3. For other SNPs, there were no significant differences between bTB-positive and control groups.

SNP rs-number	Chromosome position§	Gene feature	genotype	Gene model function§	m RNA position§	Allele change§	Protein position§	Residue change§
rs438480308	2:107117145	exon 4	CC	missense	338	CTG/ATG	89	L [Leu]/M [Met]
g.107117166A>G	2:107117166	exon 4	GG, AG	missense	359	GCC/ACC	96	A [Ala]/T [Thr]
rs458688926	2:107117167	exon 4	CC, CG	missense	360	GCC/GGC	96	A [Ala]/G [Gly]
rs478409125	2:107117180	exon 4	CC	cds-synon	373	GGC/GGG	100	G [Gly]/G [Gly]
rs440590557	2:107117280	intron 4	CC	N.D.‡	18	C/A		not found
rs460647173	2:107117291	intron 4	GG	N.D.	29	G/C		not found
rs717758716	2:107117300	intron 4	GG	N.D.	38	G/T		not found
rs480706808	2:107117321	Intron 4	AA	N.D.	59	A/T		not found
rs449522039	2:107117326	intron 4	AA, AT	N.D.	63	A/T		not found
rs469205385	2:107117328	intron 4	GG	N.D.	66	G/T		not found
g.107117369C>T	2:107117369	intron 4	TT, TC	N.D.	107	T/C		not found
rs520905988	2:107117380	intron 4	GG	N.D.	118	A/G		not found
rs482869550	2:107117389	Intron 4	AA	N.D.	127	A/C		not found
rs445253274	2:107117391	intron 4	GG	N.D.	129	G/T		not found
rs465479809	2:107117392	intron 4	GG	N.D.	130	G/T		not found
rs433744578	2:107117395	intron 4	GG	N.D.	133	G/T		not found
rs453907967	2:107117397	intron 4	GG	N.D.	135	G/T		not found
rs524557355	2:107117405	intron 4	CC	N.D.	143	C/T		not found

Table 1: Influence of genetic variants on bovine tuberculosis susceptibility in Holstein cattle. The single-nucleotide polymorphisms (SNP) are located in the exon 4 and intron 4 of bovine *SLC11A1* gene. ‡N.D.= not determined. §source: National Center for Biotechnology Information (NCBI), Database of Single Nucleotide Polymorphisms (dbSNP).

SNP*	Genotype	Allele frequency		Odds ratio (95% confidence interval)	p-value
		Infected cattle (No. /%)	Non-infected cattle (No. /%)		
g.107117166A>G	GG	50 (83.33)	85 (94.44)	1	0.048‡
	GA	10 (16.67)	5 (5.56)	3.40 (1.10-10.51)	
rs458688926	CC	53 (88.33)	86 (95.56)	1	0.12
	CG	7 (11.67)	4 (4.44)	2.84 (0.79-10.16)	
rs449522039	AA	58 (96.67)	89 (98.89)	1	0.56
	AT	2 (3.33)	1 (1.11)	3.07 (0.27-34.62)	
g.107117369C>T	TT	25 (41.67)	14 (15.56)	1	0.001‡

Table 2: Frequency distribution of genotypes of *SLC11A1* gene in the *Mycobacterium bovis* infected and non-infected Holstein cattle.*Single-nucleotide polymorphism. ‡ $p<0.05$.

SNP*	Genotype	Allele frequency		Odds ratio (95% confidence interval)	p-value
		Infected cattle (No. / %)	Non-infected cattle (No./%)		
g.107117166A>G	G	110 (91.67)	175 (97.22)	1.00	0.055
	A	10 (8.33)	5 (2.78)	3.18 (1.06-9.56)	
rs458688926	C	113 (94.17)	176 (97.78)	1.00	0.12
	G	7 (5.83)	4 (2.22)	2.73 (0.78-9.52)	
rs449522039	A	118 (93.33)	179 (99.44)	1.00	0.57
	T	2 (1.67)	1 (0.56)	3.03 (0.27-33.84)	
g.107117369C>T	T	85 (70.83)	104 (57.78)	1.00	0.030‡
	C	35 (29.17)	76 (42.22)	0.56 (0.34-0.92)	

Table 3: Frequency distribution of the alleles of *SLC11A1* gene in the *Mycobacterium bovis* infected and non-infected Holstein cattle.*Single-nucleotide polymorphism. ‡ $p<0.05$.

Discussion

This study has identified the g.107117166A>G (conservative single base A>G substitution at codon 96, resulting in a change to threonine from alanine) and g.107117369C>T (single nucleotide C>T change in intron 4) polymorphisms of the *SLC11A1* gene associated with bTB in Taiwan Holstein cattle. Similar findings have also been reported in African Zebu (3'UTR microsatellite polymorphisms of *SLC11A1* gene), Chinese Holstein cattle (G1596A SNP of *TLR1* gene) and African Buffalo (SNP41 and SNP137 polymorphisms of *SLC7A13* and *DMBT1* gene) associated with susceptibility to bTB [15,30-31]. NRAMP1 (encoded by the *SLC11A1* gene) is found in the endosome and phagosome membranes of macrophages and monocytes, and plays a pivotal role in inhibiting proliferation of *M. tuberculosis* through its involvement acidification of phagosomes. Moreover, NRAMP1 regulates the concentrations of divalent cations and nitric oxide, which confer anti-microbicidal actions and contribute to protective immune responses [4,9].

Recent findings have demonstrated significant heritable variation in susceptibility of individual Holstein cattle to bTB, supporting the importance of genetic polymorphisms in ability to control the incidence and severity of bTB outbreaks in animal herds [16,19-20]. Naturally resistant animals had the highest phagocytosis index and showed greater microbial control after exposure to *M. bovis* stimuli, producing stronger pro-inflammatory responses compared with susceptible animals [23]. In seeking to understand the reasons for natural variation in resistance, a high expression of NRAMP1 in peripheral blood cells and tuberculous granulomas from *M. bovis*-infected cattle appears to support a functional association for the *SLC11A1* gene with enhanced protection against mycobacteria [5,23]. In bovines, two *SLC11A1* alleles have been cloned and mapped to chromosome 2q43, which corresponds to a region predicted to encode a transmembrane polypeptide molecule that contains a conserved transporter motif and multiple phosphorylation sites [6,13-15]. Kadarmideen and colleagues demonstrated that the polymorphic alleles in the 3'UTR microsatellite of the *SLC11A1* gene in Zebu cattle are linked to resistance to bTB. The frequency of the 3'UTR (GT)n microsatellite of the *SLC11A1* gene is markedly different between Zebu and Holstein cattle. In Zebu cattle, GT_{13}, GT_{14}, GT_{13}/GT_{14}, and GT_{13}/GT_{15} genotypes were found, while Holstein cattle, in contrast, were homogeneous for genotype (with 100% of the animals having the GT_{13} genotype). The pathology was significantly more severe in Holstein compared with Zebu cattle, under identical husbandry conditions [16,25].

Not all studies support an association between *SLC11A1* polymorphisms and susceptibility to *M. bovis* infection. Indeed, Barthel and colleagues reported that polymorphic alleles in the 3'UTR (GT)n microsatellite of the *SLC11A1* gene do not affect resistance or susceptibility to infection by *M. bovis* in cattle [24]. Importantly, the susceptible phenotype is due to a nucleotide substitution that results in an amino acid change, rather than polymorphisms in the 3'UTR (GT)n microsatellite sequences. In addition, it is not completely clear how polymorphisms in the 3'UTR (GT)n microsatellite influence NRAMP1 function [25].

Toward understanding how polymorphisms can lead to functional consequences, Ables et al. identified two nucleotide substitutions in intron 4 of the *SLC11A1* gene in different cattle [17]. Previous studies on the INT4 polymorphism (G>C change in intron 4) have shown that exon 4a is encoded by an Alu element within intron 4, resulting in a truncated and hence non-functional NRAMP1[26]. In supporting the importance of the human *SLC11A1* gene in host defense mechanisms against tuberculosis [8], previous studies support associations between INT4 polymorphisms and bacilli growth (which may affect outcomes in pulmonary tuberculosis) [7,10,21,27]. Furthermore, variations in the promoter in linkage disequilibrium with INT4 may regulate the level of normal *SLC11A1* mRNA transcribed, and/or the ratio of normal to alternatively spliced product (for example, exon 4a–which was found to be expressed *in vivo* – would introduce a termination codon in the downstream exon 5, resulting in a severely truncated protein) [26]. Hence, we hypothesized that any variation in exon 4 and intron 4 of the bovine *SLC11A1* gene that results in a protein sequence change may influence the relative levels of normal and alternatively spliced *SLC11A1* product. To date, there are only limited data on polymorphisms in exon 4 and intron 4 of the bovine *SLC11A1* gene and bTB susceptibility or resistance.

We sought to determine if there were associations between bTB susceptibility and 4 SNP polymorphisms in exon 4 (rs438480308, rs458688926, rs478409125 and g.107117166A>G), and 14 SNPs polymorphisms in intron 4 of the *SLC11A1* gene (rs440590557, rs460647173, rs717758716, rs480706808, rs449522039, rs469205385, rs520905988, rs482869550, rs445253274, rs465479809, rs433744578, rs453907967, rs524557355 and g.107117369C>T). We observed a statistically significant difference for the g.107117166A>G SNP genotype in exon 4 of the *SLC11A1* gene, between bTB-infected and non-infected cattle (p=0.048). Those with the GA genotype had a greater relative risk of acquiring bTB than those with the GG genotype (OR: 3.40; 95% CI: 1.10–10.51). This result indicates that the GA genotype may increase susceptibility to bTB. The allele frequencies of the susceptibility loci that the A allele of g.107117166A>G polymorphism compared with the G allele was 3.18 (95% CI: 1.06–9.56*;* p=0.055) fold higher. The deduced amino acid sequence for the protein product revealed an alanine to threonine conversion at position 96 (resulting from a codon change from GCC to ACC), which may affect initiation of protein synthesis and disrupt normal NRAMP1 function that protects animals against mycobacterial infection. Previous study has reported that the G1596A SNP in the *TLR1* gene is associated with susceptibility to bTB in Chinese Holstein cattle. The reason for this association might be the conversion of isoleucine to valine, which may hinder the recognition of mycobacterial pathogen-associated molecular patterns [29-31]. A similar relationship between SNP41 and SNP137 polymorphisms in the African buffalo (*Syncerus caffer*) and bTB have been explored. SNP41 occurs in the *SLC7A13* gene, and the functional consequence is missense, from isoleucine to valine, which may impact the light chain of heteromeric amino acid transporters. SNP137 is located in the coding region of the Deleted in Malignant Brain Tumour-1 *(DMBT1*) gene. The functional consequence of SNP137 is missense, from histidine to arginine, which may the pattern recognition receptors [30]. However, the exact mechanism underlying this increased risk remains to be elucidated. Indeed, there are different levels at which increased susceptibility and resistance may be explained, such as resistance to infection or to disease progression. For the g.107117369C>T SNP polymorphism (which results in a C to T mutation in intron 4), sequence analyses indicated a lower relative risk for bTB for the TC compared with the TT genotype (OR: 0.26; 95% CI: 0.12–0.56; p=0.001). These results indicate that the TC genotype may be protective against bTB. Furthermore, the C allele of g.107117369C>T polymorphism compared with the T allele was 0.56 (95% CI: 0.34–0.92; p=0.030) fold lower. Based on previous work [17,26], the g.107117369C>T polymorphism may affect elements that control transcription and splicing of the *SLC11A1*, due to its location within the gene; in turn, this may result in incomplete or inactive proteins. Although the intron 4 polymorphism has no known functional effect, it has been suggested to be in linkage disequilibrium with functional promoter polymorphisms, which may affect pathophysiological characteristics in tuberculosis [26,28-29]. For other polymorphisms (rs438480308, rs458688926, rs478409125, rs440590557, rs460647173, rs717758716, rs480706808, rs449522039, rs469205385, rs520905988, rs482869550, rs445253274, rs465479809, rs433744578, rs453907967, and rs524557355), we found no significant associations with susceptibility to bTB-infection.

Conclusion

This is the first report to our knowledge that demonstrates the implications of polymorphisms in exon 4 and intron 4 of the *SLC11A1* gene on bTB susceptibility in cows. Our data indicate that genetic variations in *SLC11A1* exon 4 (g.107117166A>G, which results in an alanine to threonine amino acid change at position 96) and intron 4 (g. 107117369C>T) may contribute to the occurrence and development of bTB, strengthening the hypothesis that polymorphisms in this gene are associated with bTB risk in Holstein cattle. The results from this study have potential for marker-assisted breeding programs. Gene function and association studies in larger populations are still necessary to confirm these findings and to understand the biological mechanism underlying *SLC11A1*-mediated bTB susceptibility.

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