

Relationship of bovine *TNF- α* gene polymorphisms with 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* (*M. bovis*), the causative agent of zoonotic tuberculosis. *TNF- α* promotes inflammation and induces apoptosis in response to mycobacterial infection. The aim of the present study was to investigate the influence of single nucleotide polymorphisms of the *TNF- α* gene on bovine tuberculosis (bTB) susceptibility. We genotyped the *TNF- α* gene in 74 bTB-infected Holstein cows and 90 healthy control animals. The influence in the exon 3 region of *TNF- α* polymorphisms on bTB susceptibility was subsequently investigated by association analysis. Our finding demonstrated that the g.27534932A>C polymorphism of the *TNF- α* is associated with bTB in Holstein cattle. The susceptibility of cattle with the g.27534932A>C genotype compared with the CC genotype was 4.11-fold (95% CI, 1.27–13.36; $P=0.02$) higher. The g.27534932A>C polymorphism located in exon 3 of the *TNF- α* gene, and the functional consequence was missense. The deduced amino acid sequence for the protein product revealed an arginine to serine conversion at position 159, which may affect initiation of protein synthesis and disrupt normal *TNF- α* function that protects animals against mycobacterial infection. A significant association was observed with the A allele as a risk factor for bTB susceptibility (OR, 3.84; 95% CI, 1.21–12.17; $P=0.02$). In conclusion, this is the first report showing that the g.27534932A>C polymorphism may contribute to *TNF- α* -mediated bTB susceptibility.

KEY WORDS: bovine tuberculosis, polymorphism, susceptibility, *TNF- α*

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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 [2, 6, 13, 16, 19]. The worldwide prevalence of bTB remains a significant economic burden, with annual losses of approximately 3 billion US dollars [2]. Most bTB control programs rely on the intradermal tuberculin skin test (TST) to identify infected cattle, which are then culled [13]. 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 is still present in animals and humans, and the proportion of TST-positive cattle has increased from 0.13% in 2008 to 0.2% in 2014 [11]. The failure of the present measures to eradicate bTB necessitates the consideration of additional or complementary control measures.

The pathogenic state of bacterial infection and the probability of reactivation depend on the balance between host immunity and the influence of exogenous factors. During latent tuberculosis infection, *Mycobacterium* bacteria exist

in a dormant state, and through certain promoting factors, such as malnutrition and air pollution, they may later convert from the latent form into actively growing bacilli [7]. Host genetic factors have an important role in the progression of the disease. However, a dynamic relationship was proposed between the quiescent and active state having bidirectional shifts, depending on the load and virulence of the host's immune conditions [4]. Previous studies suggest that certain host genetic variants contribute to tuberculosis susceptibility [2, 3, 6, 9]. Candidate genes including tumor necrosis factor α (*TNF- α*), solute carrier family 11 member 1 (*SLC11A1*), inducible nitric oxide synthase (*iNOS*), vitamin D receptor (*VDR*), toll-like receptors (*TLRs*) and human leukocyte antigen (*HLA-DRB1* and *HLA-DQB1*) have been investigated for possible associations between genetic variants and tuberculosis susceptibility [5, 16, 20, 25, 26, 28, 38].

The balance between the Th₁ and Th₂ cytokines reflects the outcome of naïve T cell activation and assists in elucidation of the immune protection profile of the host against *Mycobacterium* bacteria. Certain cytokine gene polymorphisms also correlate with their *in vitro* cytokine secretion [15]. *TNF- α* is one of the most important pro-inflammatory and pro-immune cytokines, playing a key role in initiation, regulation and perpetuation of the inflammatory response. *TNF- α* is also required for induction of apoptosis in response to mycobacterial infection [1, 23, 40]. Many single nucleotide polymorphisms (SNPs) have been identified within the promoter of the *TNF- α* gene. Variants in the *TNF- α* promoter at positions –238G/A (rs361525), –308G/A (rs1800629), –857C/T (rs1799724) and –863A/C (rs1800630) have

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Table 1. Influence of genetic variants on bovine tuberculosis susceptibility in Holstein cattle. The single-nucleotide polymorphisms (SNPs) are located in exon 3 of the bovine *TNF- α* gene

SNP rs-number	Chromosome position ^{a)}	Genotype	Gene model function ^{a)}	mRNA position ^{a)}	Allele change ^{a)}	Protein position ^{a)}	Residue change ^{a)}
rs453652706	23:27535013	GA	missense	587	<u>C</u> TT/ <u>T</u> TT ^{b)}	132	L [Leu]/F [Phe]
rs383890418	23:27534972	CC, CT	cds-synon	628	GG <u>C</u> /GG <u>T</u>	145	G [Gly]/G [Gly]
rs432068289	23:27534962	AA, AT	missense	638	<u>A</u> CC/ <u>T</u> CC	149	T [Thr]/S [Ser]
g.27534932A>C	23:27534932	CC, CA	missense	668	<u>C</u> GC/ <u>A</u> GC	159	R [Arg]/S [Ser]

a) Source: National Center for Biotechnology Information (NCBI), the Single Nucleotide Polymorphism database (<http://www.ncbi.nlm.nih.gov/snp>). b) SNP to mRNA is reverse strand.

been examined for their associations with susceptibility or resistance to tuberculosis [1, 5, 26, 28]. The rs361525 and rs1800629 polymorphisms were found to be associated with pulmonary tuberculosis in Colombian and Iranian populations. The associations between TB and the rs1799724 and rs180063 polymorphisms have also been documented in the Chinese Han population [33]. The most important of these SNPs, rs1800629, is associated with variable levels of pro-inflammatory cytokines and the pathogenesis of tuberculosis. However, little information is available regarding the associations between bTB susceptibility and gene mutations. Thus, we aimed to identify possible associations between *TNF- α* polymorphisms and the risk of bTB in Holstein cattle.

MATERIALS AND METHODS

Animals of study: Our cohort comprised 164 Holstein cattle in Taiwan (age range: 0.7–5.0 years), 74 of which were mandatorily sent to be culled between January 2011 and October of 2015 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 case-control study.

Preparation of bovine genomic DNA: For extracting DNA, 5–10 ml of peripheral blood was collected from each subject, stored at -20°C and taken to the laboratory on dry ice. DNA from the blood samples was extracted using DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD, U.S.A.), according to the manufacturer's instructions. The concentration and purity of the extracted DNA was verified optically using an ND-1000 spectrophotometer (NanoDrop Technology, Wilmington, DL, U.S.A.).

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 $10\times$ 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'-CCT GCT GAC GGG TTT ACC T-3', and reverse, 5'-ATG GCA GAC AGG ATG TTG ACC-3', primers were used for amplifying a *TNF- α* fragment as described previously [40]. The reaction was initiated by heating the mixture to 94°C for 5 min, followed by 35 cycles at 94°C for 50 sec, 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 142 base pairs, products were then sequenced using a BigDye terminator cycle sequencing kit in an Applied Biosystems 3730 \times 1 DNA Analyzer (Applied Biosystems, Waltham, MA, U.S.A.), using the abovementioned PCR primers.

Statistical methods: The chi-square test was used to examine the association between genetic polymorphisms and susceptibility to infection in animals. 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, the heterogeneity of odds ratios (ORs) for susceptibility to bTB infection was assessed. ORs and 95% confidence intervals (CIs) were calculated in order to estimate risk of disease in animals. Statistical significance was set at 5% ($P<0.05$).

RESULTS

Polymorphisms in exon 3 of the bovine *TNF- α* gene: The SNPs including rs453652706, rs383890418, rs432068289 and g.27534932A>C were identified in exon 3 of the bovine *TNF- α* gene. Only one genotype, GA, of the 453652706 polymorphism was found in both bTB-positive and control cattle. For this reason, we did not include the rs453652706 SNP in the results. Two genotypes, CC and CT, of the rs383890418 polymorphism were found in both bTB-positive and control cattle. Two genotypes, AA and AT, of the rs432068289 polymorphism were also found. For the g.27534932A>C polymorphism, two genotypes, CC and CA, were identified. The influence of genetic variants on bTB susceptibility is presented in Tables 1 and 2. Of the bTB group ($n=74$), 83.78% ($n=62$) had the homozygous CC genotype at the

Table 2. Frequency distribution of genotypes of the *TNF- α* gene in the *Mycobacterium bovis* infected and non-infected Holstein cattle

SNP ^{a)}	Genotype	Genotype frequency		Odds ratio (95% confidence interval)	P-value
		Infected cattle (No. /%)	Non-infected cattle (No. /%)		
rs383890418	CC	50 (67.57)	68 (75.56)	1	0.30
	CT	24 (32.43)	22 (24.44)	1.48 (0.75–2.94)	
rs432068289	AA	73 (98.65)	85 (94.44)	1	0.22
	AT	1 (1.35)	5 (5.56)	0.23 (0.03–2.04)	
g.27534932A>C	CC	62 (83.78)	85 (95.51)	1	0.02 ^{b)}
	CA	12 (16.22)	4 (4.49)	4.11 (1.27–13.36)	

a) Single nucleotide polymorphism. b) $P < 0.05$.

Table 3. Frequency distribution of the alleles of the *TNF- α* gene in the *Mycobacterium bovis* infected and non-infected Holstein cattle

SNP ^{a)}	Genotype	Allele frequency		Odds ratio (95% confidence interval)	P-value
		Infected cattle (No. /%)	Non-infected cattle (No. /%)		
rs383890418	C	124 (83.78)	158 (87.78)	1	0.63
	T	24 (16.22)	22 (12.22)	1.39 (0.74–2.60)	
rs432068289	A	147 (99.32)	175 (97.22)	1	0.23
	T	1 (0.68)	5 (2.78)	0.24 (0.03–2.06)	
g.27534932A>C	C	136 (91.89)	174 (97.75)	1	0.02 ^{b)}
	A	12 (8.11)	4 (2.25)	3.84 (1.21–12.17)	

a) Single nucleotide polymorphism. b) $P < 0.05$.

polymorphism, and 16.22% ($n=12$) had the heterozygous CA genotype at the polymorphism. Of the control group ($n=90$), 95.51% ($n=85$) had the CC genotype at the SNP, and 4.49% ($n=4$) had the CA genotype at the SNP (Table 2); the frequencies of the high-producing A allele revealed that it was overrepresented in the bTB group compared with the control group (8.11% versus 2.25%) (Table 3).

Effects of the polymorphisms on bTB risk: The polymorphism g.27534932A>C of the *TNF- α* gene was significantly associated with bTB in Holstein cattle. The susceptibility of cattle with the g.27534932A>C genotype compared with the CC genotype was 4.11-fold (95% CI, 1.27–13.36; $P=0.02$) higher. Furthermore, the A allele was a risk factor for bTB susceptibility (OR, 3.84; 95% CI, 1.21–12.17; $P=0.02$). The results demonstrated the effect of g.27534932A>C polymorphisms of the *TNF- α* gene on bTB susceptibility in Holstein cattle. The rs383890418 SNP produced two different conformation patterns (CC and CT), and the CT genotype was overrepresented in the bTB group (32.43%) compared with the control group (24.44%). The CT genotype frequency of the rs383890418 variant was not significantly higher in bTB cattle than in healthy controls (OR, 1.48; 95% CI, 0.75–2.94; $P=0.30$). There was no significant association between the rs432068289 polymorphism and bTB resistance ($P=0.22$). The AT genotypes had a lower relative risk of bTB (OR, 0.23; 95% CI, 0.03–2.04) as compared with the AA genotype. The genotype and allelic distributions of infected and control animals are presented in Tables 2 and 3.

DISCUSSION

This study identified the g.27534932A>C (conservative single nucleotide adenine to cytosine substitution at codon 159, resulting in a change to serine from arginine) polymorphism of the *TNF- α* gene associated with bTB in Holstein cattle in Taiwan and genetic variations similar to some found in African zebu (3'UTR microsatellite polymorphisms of the *SLC11A1* gene), Chinese Holstein cattle (G1596A SNP of the *TLR1* gene) and African buffalo (SNP41 and SNP137 polymorphisms of the *SLC7A13* and *DMBT1* gene) associated with susceptibility to bTB [20, 32, 35].

Recent findings have demonstrated significant heritable differences in the susceptibility of Holstein cattle to bTB, supporting the importance of genetic polymorphisms in ability to control the incidence and severity of bTB outbreaks in animal herds [8, 12]. A previous study reported that the G1596A SNP in the *TLR1* gene is associated with susceptibility to bTB in Holstein cattle (*Bos taurus*). The reason for this association might be the conversion of isoleucine to valine, which may hinder the recognition of mycobacterial pathogen-associated molecular patterns [31]. A similar relationship between SNP41 and SNP137 polymorphisms in the African buffalo (*Syncerus caffer*) and bTB has 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 *DMBT1* gene. The functional consequence of SNP137 is

missense, from histidine to arginine, which may impact the pattern recognition receptors [27]. A Chadian study showed that 3'UTR microsatellite polymorphisms of the *SLC11A1* gene were associated with susceptibility to bTB in African zebu (*Bos indicus*) [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 [10]. *TNF- α* is essential for the formation of protective tuberculosis granulomas and regulates the expression of other cytokines that contribute to a protective immune response and exert antimicrobial action [1, 28, 40]. In seeking to understand the reasons for natural variation in resistance, high expression of the *TNF- α* gene in bovine macrophages in response to challenge with *M. bovis* appears to support a functional association for the *TNF- α* gene with enhanced protection against mycobacteria [27, 40]. In bovines, the *TNF- α* gene contains 4 exons and 3 introns and is located on chromosome 23q22 [24]. In the past decade, several studies have investigated the relationship between polymorphisms in the *TNF- α* gene and susceptibility to clinical mastitis, immune function and bovine leukemia virus infection [8, 21, 22, 30, 37, 39], but little is known about the role of the *TNF- α* gene in bTB.

Regarding the current understanding of how polymorphisms can lead to functional consequences, previous studies have reported that the polymorphisms in human *TNF- α* have revealed the importance of this protein in human defense against tuberculosis. The rs361525, rs1800629 and rs1800630 SNPs in the *TNF- α* gene have been examined for their associations with susceptibility or resistance to tuberculosis [5, 33, 34, 36]. It has been observed that the rs361525 and rs1800629 SNPs are the most important in human tuberculosis susceptibility, as they might influence transcription of the *TNF- α* gene [29]. Regarding the rs1800629 SNP (a guanine to adenine nucleotide change), allele A (the minor allele) reportedly increases the rate of gene transcription and has been associated with increased inflammatory response [31]. The cytokines thus released in a paracrine manner in serum may also provide an indirect measure of cytokine function. The AG genotype of the rs1800629 SNP showed high levels of the *TNF- α* protein in serum in active pulmonary tuberculosis patients when compared with healthy controls. The association between the SNPs and their cytokine production indicate the functional importance of cytokines in gene expression [18]. The rs1800630 SNP (an adenine to cytosine nucleotide change) appears to be associated with an increase in the lung function tests considering a ratio of forced expiratory volume in 1 second/forced vital capacity (FEV1/FVC) was identified [31]. Possible evolutionary selection pressure appears to affect *TNF- α* gene expression rather than protein structure, a common finding among the tuberculosis susceptibility genes, as regulation of gene expression may be a prevalent selection mechanism in evolution. Evaluation of the cytokines and any of their inherent polymorphisms might provide a useful diagnostic tool in evaluating immune regulation and progression of the disease [18]. Hence, we hypothesized that any variation in exon 3

of the bovine *TNF- α* gene that results in a protein sequence change may influence the relative levels of normal and alternatively *TNF- α* products. To date, there are only limited data on polymorphisms in exon 3 of the bovine *TNF- α* gene and bTB susceptibility or resistance.

We sought to determine if there were associations between bTB susceptibility and 4 SNPs in exon 3 (rs453652706, rs383890418, rs432068289 and g.27534932A>C) of the *TNF- α* gene. For the g.27534932A>C polymorphism, two genotypes, CC and CA, were found. We observed a statistically significant difference in the g.27534932A>C SNP genotype of bTB-infected and noninfected cattle ($P=0.02$), with those carrying the CA genotype having a greater relative risk of acquiring bTB than those with the CC genotype (OR, 4.11; 95% CI, 1.27–13.36). In the present study, we also observed that the rates of occurrence of bTB in animals with the CA genotype in the infected groups (16.22%) were higher compared with in the other groups (4.49%). These results indicate that the CA genotype is more likely susceptible to bTB. Furthermore, the risk of bTB was 3.84-fold higher for individuals carrying the A allele of g.27534932A>C SNP than those carrying the C allele (95% CI, 1.21–12.17; $P=0.02$). Multiple studies have revealed that SNPs in exons of the *TNF- α* gene affect the mRNA expression of *TNF- α* and immune function in dairy cows, and allele A of the gene encoding *TNF- α* , whose role is to activate the immune system during infection, proved to be very beneficial—it is linked to higher immunity against infection compared with the other allele in this locus [21, 30, 37]. The g.27534932A>C SNP belongs to the non-synonymous SNPs (nsSNPs), also called missense variants, which are particularly important, as they result in changes in the translated amino acid residue sequence. nsSNPs may affect protein function by reducing protein solubility or by destabilizing the protein structure, and they may affect gene regulation by altering transcription and translation [12]. The deduced amino acid sequence of the g.27534932A>C SNP had one arginine (Arg) to serine (Ser) conversion at position 159 (codon change to AGC from CGC), which may hinder the initiation and regulation of the inflammatory response against mycobacterial infection. It has been found that *TNF- α* enhancer polymorphism influences the serum level of *TNF- α* in tuberculosis and thus affects the susceptibility to diseases. The presence of DNA sequence variation in the *TNF- α* gene causes modification of transcriptional regulation and thus is responsible for the association with susceptibility/resistance to tuberculosis [29]. Dabhi *et al.* suggested that rs4645843 (proline to leucine conversion at position 84) and rs1800620 (alanine to threonine conversion at position 94) variants of *TNF- α* could directly or indirectly destabilize the amino acid interactions and hydrogen bond networks, thus explaining the functional deviations of protein to some extent. Protein structural analysis with the above variants was performed by using I-Mutant and MUSTER (MUlti-Sources ThreadER) servers to check their molecular dynamics and energy minimization calculations. They were found to have decreased the stability of the protein structure. This may be due to a mutant residue that is bigger than the wild type and cannot fit within the

available space. The two identified *TNF- α* variants could disrupt regulation of the inflammatory signaling cascade and thus may participate in inflammatory diseases [12]. The production of *TNF- α* is regulated at the transcriptional and translational level [17]. It is postulated that mutations in the cytokine genes may influence the level of cytokine production and thus the elicited dysregulated immune response [14]. Hence, the g.27534932A>C (Arg159Ser) variants constitute a unique resource of genetic markers that may considerably increase the power of screening for mutations in the *TNF- α* gene in bTB epidemiological studies. 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 other polymorphisms (rs453652706, rs383890418 and rs432068289), we found no significant associations with susceptibility to bTB infection.

In conclusion, the present study revealed that genetic variations in *TNF- α* exon 3 (g.27534932A>C SNP, Arg-159Ser) may contribute to the occurrence and development of bTB, strengthening the hypothesis that polymorphisms of the *TNF- α* gene are associated with risk of bTB in Holstein cattle. To the best of our knowledge, this is the first report regarding the impact of polymorphisms in *TNF- α* on bTB susceptibility in cows. Gene function and association studies of a larger population are still necessary to confirm these findings and to investigate the biological mechanism underlying *TNF- α* -mediated bTB susceptibility.

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