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Running Head: NOS2 ASSOCIATED WITH BOVINE TUBERCULOSIS

Relationship of bovine NOS2 gene 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 inducible nitric oxide synthase (iNOS which is encoded by the *NOS2* gene) plays a key role in the immunological control of a broad spectrum of infectious agents. This study aimed to investigate the influence of genetic variations in the promoter of the *NOS2* gene on bovine tuberculosis (bTB) susceptibility. In this study, the *NOS2* genes of 74 bTB-infected Holstein cows and 90 healthy controls were genotyped using PCR followed by nucleotide sequencing. Polymorphisms at rs207692718, rs109279434, rs209895548, rs385993919, rs433717754, rs383366213, rs466730386, rs715225976, rs525673647, rs720757654 and g.19958101T > G in the promoter region of the *NOS2* gene were detected. The g.19958101T > G SNP produced two different conformation patterns (TT and TG) and the TG genotype was over-represented in the bTB group (20.27%) compared with the control group (2.22%). The TG genotype frequency of the g.19958101T > G variant was significantly higher in bTB cattle than in healthy controls (OR, 11.19; 95% CI, 2.47–50.73; p = 0.0002). The G allele of the g.19958101T > G polymorphism was more frequent in bTB group when compared to control group (10.14% versus 1.11%). Furthermore, the G allele was a risk factor for bTB susceptibility (OR, 10.04; 95% CI, 2.26–44.65; p = 0.0002). In conclusion, the g.19958101T > G polymorphism of the *NOS2* gene may contribute to the susceptibility of Holstein cattle to bTB.

KEY WORDS: bovine tuberculosis, *NOS2*, susceptibility
*Mycobacterium bovis* (*M. bovis*) is the main causative agent of bovine tuberculosis (*bTB*), a disease of great concern in many countries, including England, Ireland, Brazil, China and Taiwan, for its detrimental economic impact and its effect on humans [3, 4, 8, 13, 16, 18]. The worldwide prevalence of *bTB* remains a significant economic burden, with annual losses of approximately 3 billion US dollars [1]. To control *bTB*, most programs rely on intradermal tuberculin skin test (TST) to identify infected cattle, which are then sacrificed [30]. In Taiwan, a national *bTB* eradication comprising annual TST, restriction of animal movement and slaughter of reactor animals, has been implemented since 1947. Despite these efforts, animal and human tuberculosis are still present. The rate of TST-positive cattle increased from 0.22% in 2000 to 0.36% in 2002 [13]. Failure of the present measures to eradicate *bTB* necessitates the consideration of additional or complementary control measures.

Previous studies suggest that certain host genetic variants contribute to tuberculosis susceptibility [1-4]. Several candidate proteins associated with the susceptibility, including nitric oxide synthase 2 inducible gene (*NOS2*), tumor necrosis factor α (*TNF-α*), solute carrier family 11 member 1 (*SLC11A1*), vitamin D receptor (*VDR*) and toll-like receptors (*TLRs*), have been investigated for their possible associations between genetic variants and tuberculosis susceptibility [11, 15, 16, 18, 19, 23, 31, 35]. The inducible nitric oxide synthase (iNOS) which is encoded by the *NOS2* gene is a cytoplasmic protein and absent in resting cells, but is rapidly produced in response to stimuli, such as infections and cytokines [6, 12, 21]. The iNOS synthesizes nitric oxide which has both cytotoxic and cytoprotective effects. Nitric oxide is vital for macrophage function and granuloma formation in the immune response and kills *M. tuberculosis* *in vitro* [7, 9, 22]. Pereira-Suárez et al. demonstrate the expression of iNOS is stimulated in granulomas, which are protective T-cell reactions against mycobacteria [25]. Investigation of this possibility is hampered by difficulty in estimating the production of nitric oxide *in vivo* mainly in lung tissues, but genetic analysis opens a window to study the possible relations between *NOS2* expression and consequences.
of tuberculosis [16, 20]. NOS2 genetic defects in both transcriptional and posttranscriptional regulatory functions may contribute to the susceptibility to TB progression [35].

Many single nucleotide polymorphisms (SNPs) have been identified within the promoter of NOS2 gene that is linked to increased enzyme expression, resulting in higher nitric oxide production. The rs2779249 and rs2301369 SNPs have been found to be associated with tuberculosis in Brazilians. The rs2779249, rs9282799 and rs8078340 SNPs are associated with tuberculosis in South African colored population [21]. On the other hand, the rs2297518 SNP in exon 16 may be an important protective factor to pulmonary tuberculosis in Chinese miners [29]. In addition, the rs2274894, rs57234985 and rs7215373 SNPs are believed to associate with tuberculosis in African American population [35]. However, little information is available regarding the association between bTB susceptibility and gene mutations. Thus, in the present study, we aimed to identify possible associations between NOS2 promoter variants and the risk of bTB in Holstein cattle.

MATERIALS AND METHODS

Animals of study: Our cohort comprised 164 Holstein cattle in Taiwan, 74 (age range: 0.7–5.0 years) of which were mandatorily sent to slaughterhouses after a positive reaction for the TST following Taiwan regulations during 2011 to 2015. The 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 here were reviewed and approved by the National Taiwan University Institutional Animal Care and Use Committee. Subjects with a M. bovis-positive lymph node culture were classified as having bacteria-positive bTB.

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 in dry ice. DNA from the blood samples was extracted using DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD, USA),
following the manufacturer's instructions. Concentration and purity of the extracted DNA was verified optically by ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, DL, USA).

Genotyping by polymerase chain reaction (PCR): The SNPs in the promoter region of bovine NOS2 gene (base pair at 19958047-19958447 position of bovine chromosome 19) were analyzed by PCR followed by DNA sequencing. The following SNPs in order of the 5’end of NOS2 gene which were looking for the Database of Single Nucleotide Polymorphisms (dbSNP) of the National Center for Biotechnology Information (NCBI) were detected: rs207692718, rs109279434, rs209895548, rs109279434, rs209895548, rs383366213, rs466730386, rs715225976, rs525673647, rs720757654 and g.19958101T > G. Samples for PCR were prepared in a volume of 30 μl containing 19.5 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 dNTP (Viogene Biotek Corp., New Taipei City, Taiwan), 0.5 μl of 2 unit/μl Taq DNA polymerase (Viogene Biotek Corp.) and 4 μl of the extracted DNA. The forward 5'-AGT CAC TCA GAG GCG AGT CA-3' and reverse 5'- GCC AAA CCT CAT GTT GGC AT-3' primers were used for amplifying NOS2 fragments. These primers were designed using Primer Premier 5 software according to the sequence at positions 5230-5630 in promoter region of the bovine NOS2 gene (GenBank AF333248.1). The reaction was initiated by heating at 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, and concluded by a final extension step at 72°C for 10 min. The PCR products were analyzed using 3% agarose gel electrophoresis. The product with the predicted size of 400 base pairs was then sequenced using BigDye Terminator Cycle Sequencing in an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Waltham, MA, USA) with the same primers as the corresponding PCR.

Statistical methods: The chi-square test was used to examine the association between genetic polymorphisms and individual susceptibility to the disease. 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 (OR) for susceptibility to bTB infection was assessed. The OR and 95% confidence intervals (CI) were calculated to estimate the individual risk of disease. Significance was set at 5% ($p<0.05$).

**RESULTS**

*Polymorphisms in promoter region of the bovine NOS2 gene:* The SNPs (relative to the NOS2 transcription start site) including g.19958101T > G (base pair at 19958101 position of bovine chromosome 19), rs207692718 (base pair at 19958092 position), rs109279434 (base pair at 19958183 position), rs209895548 (base pair at 19958197 position) and rs525673647 (base pair at 19958406 position) were identified in the promoter region of the NOS2 gene. The g.19958101T > G SNP produced two different conformation patterns (TT and TG). Of the bTB group (n=74), 79.73% (n=59) were homozygous TT genotype, and 20.27% (n=15) were heterozygous TG genotype at the polymorphism. 88 of 90 (97.78%) of the control group were TT genotype, and two (2.22%) were TG genotype at the SNP (Table 1). For g.19958101T > G promoter polymorphism analysis, the G allele was significantly more frequent in bTB group when compared to control group (10.14% versus 1.11%, $p=0.0002$) (Table 2). The GG, GC and CC genotypes of the rs207692718 polymorphism were found, and the C allele was over-represented in the control group (70.56%) compared with the bTB group (60.14%). Among the three genotypes of the rs109279434 polymorphisms, the CC genotype was more frequent in the bTB (87.74%) and control (91.11%) groups in comparison to GC and GG genotypes. For the rs209895548 SNP (CC, CT and TT), the T allele was over-represented in the control group (57.22%) compared with the bTB group (47.30%). Two genotypes of the rs525673647 polymorphism were identified; frequencies of high producing T allele were over-represented in bTB groups in comparison to
control group (3.38% versus 0.56%). None of the rs720757654, rs715225976, rs466730386, rs383366213, rs433717754 and rs385993919 SNPs was associated with bTB.

**Effects of the polymorphisms on bTB risk:** The g.19958101T > G polymorphism of the NOS2 gene was significantly associated with bTB in Holstein cattle. The susceptibility of cattle with the g.19958101T > G genotype compared with the TT genotype was 11.19 (95% CI, 2.47–50.73; p= 0.0002) fold higher. Furthermore, the G allele was a risk factor for bTB susceptibility (OR, 10.04; 95% CI, 2.26–44.65; p = 0.0002). The results demonstrated the implications of g.19958101T > G polymorphism of the NOS2 gene on bTB susceptibility in Holstein cattle. The association between the rs207692718, rs109279434, rs209895548 and rs525673647 polymorphisms and bTB resistance was not significant. The CT genotype of rs525673647 SNP had a higher relative risk of bTB as compared with the CC genotype (OR, 6.45; 95% CI, 0.74–56.48; p = 0.09). There was still a strong linkage between C allele of rs207692718 SNP and low risk of bTB in cattle (OR, 0.63; 95% CI, 0.40–1.00; p = 0.06). The risk of bTB for individuals carrying GC and CC genotypes of the rs109279434 SNP was 0.68-fold (95% CI, 0.22–2.13; p= 0.57) lower than those carrying GG genotype. The T allele of rs209895548 SNP was a low risk factor for predisposition to bTB (OR, 0.67, 95% CI, 0.43–1.04; p = 0.09). The genotype and allelic distributions of the cases and controls are presented in Tables 1 and 2. There was no significant difference between bTB cases and controls in other SNPs.

**DISCUSSION**

Our results indicate association between bTB incidence and the g.19958101T > G polymorphism of the NOS2 gene in Taiwan Holstein cattle, and genetic variations similar to some found 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) associate with susceptibility to bTB [14, 27, 33].
In the past decade, several studies have investigated the relationship between expression of the iNOS and bTB [6, 10, 25, 32], but little is known about the role of NOS2 polymorphisms in bTB. Recent findings have demonstrated significant heritable differences in the susceptibility of Holstein cattle to bTB [3, 4]. NOS2 gene is essential for the regulation of nitric oxide which helps killing or limiting the growth of *M. tuberculosis* and amplifying synthesis of TNF-α and IL-1β that contribute to a protective immune response and exert anti-microbicidal action [9, 16, 21]. Because NOS2-mediated nitric oxide production is primarily regulated at the transcriptional level, studies of genetic determinants of nitric oxide expression have focused on the promoter region of the NOS2 gene [17, 26]. Mutations and polymorphisms in promoter region of NOS2 gene have revealed the importance of this protein in human defense against tuberculosis [9, 20, 21, 29, 35, 36]. Two promoter mutations of NOS2 gene, rs1800482 (a cytosine to guanine nucleotide change) and rs9282799 (a guanine to adenine nucleotide change) SNPs, associate with increased nitric oxide production via the constitutively high baseline level of iNOS activity [15]. The rs57234985 (a thymine to cytosine nucleotide change) SNP is associated with low exhaled nitric oxide levels [17, 35]. The rs2779249 (a cytosine to adenine nucleotide change) SNP, for which no function has been determined as yet, is associated with tuberculosis. The rs8078340 (a guanine to adenine nucleotide change) SNP is able to decrease the quantity of DNA-protein complex bound and lead to nitric oxide reduction [9, 21, 35]. The minor (A) allele of the rs8078340 polymorphism is associated with considerably decreased affinity for nuclear protein(s), suggesting that it has functional significance [5]. Hence, we hypothesized that any variation in promoter of the bovine NOS2 gene may influence bTB susceptibility or resistance. Thus, we investigated the association between bTB susceptibility and NOS2 polymorphisms at the 11 SNPs (rs207692718, rs109279434, rs209895548, rs385993919, rs433717754, rs383366213, rs466730386, rs715225976, rs525673647, rs720757654 and g.19958101T > G) in the promoter of the NOS2 gene.

Our study found that the Holstein cattle carrying the g.19958101T > G polymorphism may be more susceptible to bTB. A statistically significant difference in the g.19958101T > G
polymorphism of bTB-infected and non-infected cattle was observed (p = 0.0002), which is carrying the TG genotype having a greater relative risk of acquiring bTB than those with the TT genotype (OR, 11.19; 95% CI, 2.47–50.73). The risk of bTB was 10.04-fold higher for individuals carrying the G allele of g.19958101T > G SNP than those carrying the T allele (95% CI, 2.26–44.65; p = 0.0002). This genetic variant conversion at the NOS2 promoter, the region of the NOS2 transcription start site, is characterized by the presence of DNA elements associated with recombination and translocation and as such likely serves an important function. The NOS2 promoter contains transcription factor binding sites including activator protein 1 (AP-1) and signal transducers and activators of transcription protein 1 (STAT-1) and involves in lipopolysaccharide or cytokine mediated transcription induction that provides basal nitric oxide levels in the absence of infection [17, 28, 34]. Nitric oxide is not just a cytotoxic molecule but also an important regulator of the Th1/Th2 balance limiting the Th1 response, which is consistent with the observation that mice with a disrupted NOS2 gene exhibit enhanced Th1 reactivity [7, 24]. The g.19958101T > G polymorphism, as well as previously shown, might have reduced the quantity of DNA-protein complex bound to this DNA region, and it is possible that individuals with the SNP may have fewer transcripts and therefore produce less nitric oxide for binding duration [21]. The thymine to guanine nucleotide change predicts the creation of a new sequence recognition site for the transcription factors. These factors, proteins that bind to specific DNA sequences and affect transcription of mRNA from DNA, could potentially account for a decreased degree of transcription from the g.19958101T > G promoter. This could be advantageous to M. bovis, because that the animals would not resist infection. However, the exact mechanism involved needs to be verified. We found no significant difference between bTB-infected and non-infected cattle with respect to the other polymorphisms.

In conclusion, the present study revealed that the g.19958101T > G polymorphism of the NOS2 gene may contribute to the occurrence and development of bTB, strengthening the hypothesis that polymorphisms of the NOS2 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 NOS2 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 NOS2-mediated bTB susceptibility.

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Table 1. Frequency distribution of the genotypes of nitric oxide synthase 2 gene in the *Mycobacterium bovis* infected and non-infected Holstein cattle.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>Genotype frequency</th>
<th>Odds ratio (95% confidence interval)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Infected cattle (No. / %)</td>
<td>Non-infected cattle (No. / %)</td>
<td></td>
</tr>
<tr>
<td>rs207692718</td>
<td>GG</td>
<td>20 (27.03)</td>
<td>15 (16.67)</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>19 (25.68)</td>
<td>23 (25.56)</td>
<td>0.62 (0.25-1.53)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>35 (47.30)</td>
<td>52 (57.78)</td>
<td>0.50 (0.23-1.12)</td>
</tr>
<tr>
<td></td>
<td>GC+CC</td>
<td>54 (72.97)</td>
<td>75 (83.33)</td>
<td>0.54 (0.25-1.15)</td>
</tr>
<tr>
<td>g.19958101T &gt; G</td>
<td>TT</td>
<td>59 (79.73)</td>
<td>88 (97.78)</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>TG</td>
<td>15 (20.27)</td>
<td>2 (2.22)</td>
<td>11.19 (2.47-50.73)</td>
</tr>
<tr>
<td>rs109279434</td>
<td>GG</td>
<td>7 (9.46)</td>
<td>6 (6.67)</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>2 (2.70)</td>
<td>2 (2.22)</td>
<td>0.86 (0.09-8.07)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>65 (87.84)</td>
<td>82 (91.11)</td>
<td>0.68 (0.22-2.12)</td>
</tr>
<tr>
<td></td>
<td>GC+CC</td>
<td>67 (90.54)</td>
<td>84 (97.78)</td>
<td>0.68 (0.22-2.13)</td>
</tr>
<tr>
<td>rs209895548</td>
<td>CC</td>
<td>36 (48.65)</td>
<td>33 (36.67)</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>6 (8.11)</td>
<td>11 (12.22)</td>
<td>0.50 (0.17-1.50)</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>32 (43.24)</td>
<td>46 (51.11)</td>
<td>0.64 (0.33-1.23)</td>
</tr>
<tr>
<td></td>
<td>CT+TT</td>
<td>38 (51.35)</td>
<td>57 (63.33)</td>
<td>0.61 (0.33-1.14)</td>
</tr>
<tr>
<td>rs525673647</td>
<td>CC</td>
<td>69 (93.24)</td>
<td>89 (98.89)</td>
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</tr>
<tr>
<td></td>
<td>CT</td>
<td>5 (6.67)</td>
<td>1 (1.11)</td>
<td>6.45 (0.74-56.48)</td>
</tr>
</tbody>
</table>

a Single-nucleotide polymorphism
b p < 0.05
Table 2. Frequency distribution of the alleles of nitric oxide synthase 2 gene in the *Mycobacterium bovis* infected and non-infected Holstein cattle.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>Infected cattle (No. / %)</th>
<th>Non-infected cattle (No. / %)</th>
<th>Odds ratio  (95% confidence interval)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs207692718</td>
<td>G</td>
<td>59 (39.86)</td>
<td>53 (29.44)</td>
<td>1.00</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>89 (60.14)</td>
<td>127 (70.56)</td>
<td>0.63 (0.40-1.00)</td>
<td>0.06</td>
</tr>
<tr>
<td>g.19958101T &gt; G</td>
<td>T</td>
<td>133 (89.86)</td>
<td>178 (98.89)</td>
<td>1.00</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>15 (10.14)</td>
<td>2 (1.11)</td>
<td>10.04 (2.26-44.65)</td>
<td>0.0002</td>
</tr>
<tr>
<td>rs109279434</td>
<td>G</td>
<td>16 (10.81)</td>
<td>14 (7.78)</td>
<td>1.00</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>132 (89.19)</td>
<td>166 (92.22)</td>
<td>0.70 (0.33-1.48)</td>
<td>0.44</td>
</tr>
<tr>
<td>rs209895548</td>
<td>C</td>
<td>78 (52.70)</td>
<td>77 (42.78)</td>
<td>1.00</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>70 (47.30)</td>
<td>103 (57.22)</td>
<td>0.67 (0.43-1.04)</td>
<td>0.09</td>
</tr>
<tr>
<td>rs525673647</td>
<td>C</td>
<td>143 (96.62)</td>
<td>179 (99.44)</td>
<td>1.00</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>5 (3.38)</td>
<td>1 (0.56)</td>
<td>6.26 (0.72-54.18)</td>
<td>0.09</td>
</tr>
</tbody>
</table>

*a* Single-nucleotide polymorphism  
*b* p < 0.05