FULL PAPER *Public Health*

Relationship of bovine *NOS2* gene polymorphisms to the risk of bovine tuberculosis in Holstein cattle

Yafen CHENG[1,2], ChenShen HUANG[1,3] and Hsiang-Jung TSAI[1,3]*

[1]*School of Veterinary Medicine, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd., Taipei 10617, Taiwan*
[2]*Centers for Disease Control, Ministry of Health and Welfare, No. 6, Linsen S. Rd., Taipei 10050, Taiwan*
[3]*Animal Health Research Institute, Council of Agriculture, Executive Yuan, No. 376, Zhongzheng Rd., New Taipei City 25158, Taiwan*

(Received 18 May 2015/Accepted 30 September 2015/Published online in J-STAGE 15 October 2015)

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

doi: 10.1292/jvms.15-0295; *J. Vet. Med. Sci.* 78(2): 281–286, 2016

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

*CORRESPONDENCE TO: TSAI, H.-J., School of Veterinary Medicine, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd., Taipei 10617, Taiwan. e-mail: tsaihj@ntu.edu.tw

©2016 The Japanese Society of Veterinary Science
This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <http://creativecommons.org/licenses/by-nc-nd/3.0/>.

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 m*l* 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, U.S.A.), following the manufacturer's instructions. Concentration and purity of the extracted DNA was verified optically by ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, DL, U.S.A.).

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, rs385993919, rs433717754, 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 *μ*M), 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 3730 × l DNA Analyzer (Applied Biosystems, Waltham, MA, U.S.A.) 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.

Table 1. Frequency distribution of the genotypes of nitric oxide synthase 2 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. /%)		
rs207692718	GG	20 (27.03)	15 (16.67)	1.00	
	GC	19 (25.68)	23 (25.56)	0.62 (0.25–1.53)	0.36
	CC	35 (47.30)	52 (57.78)	0.50 (0.23–1.12)	0.11
	GC+CC	54 (72.97)	75 (83.33)	0.54 (0.25–1.15)	0.13
g.19958101T>G	TT	59 (79.73)	88 (97.78)	1.00	0.0002[b)]
	TG	15 (20.27)	2 (2.22)	11.19 (2.47–50.73)	
rs109279434	GG	7 (9.46)	6 (6.67)	1.00	
	GC	2 (2.70)	2 (2.22)	0.86 (0.09–8.07)	1
	CC	65 (87.84)	82 (91.11)	0.68 (0.22–2.12)	0.57
	GC+CC	67 (90.54)	84 (97.78)	0.68 (0.22–2.13)	0.57
rs209895548	CC	36 (48.65)	33 (36.67)	1.00	
	CT	6 (8.11)	11 (12.22)	0.50 (0.17–1.50)	0.43
	TT	32 (43.24)	46 (51.11)	0.64 (0.33–1.23)	0.24
	CT+TT	38 (51.35)	57 (63.33)	0.61 (0.33–1.14)	0.17
rs525673647	CC	69 (93.24)	89 (98.89)	1.00	0.09
	CT	5 (6.67)	1 (1.11)	6.45 (0.74–56.48)	

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

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

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

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 poly-

morphisms 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 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.

ACKNOWLEDGMENTS. The authors thank the members (Chian-Ren Jeng, Dah-Jiun Fu, Hsiu-Luan Chang, Meng-Chieh Hsu, Ming-Chang Lee and Shyh-Shyan Liu) who helped to collect the samples. We also thank the researchers (Albert Taiching Liao, Chia-Lan Wang, Chung-Hsi Chou, Fan Lee, Yang-Chi Chiea Fan and Yu-Chun Liu) for professional help. This research was supported by a grant from the Centers for Disease Control, Taiwan, Republic of China.

REFERENCES

1. Allen, A. R., Minozzi, G., Glass, E. J., Skuce, R. A., McDowell, S. W. J., Woolliams, J. A. and Bishop, S. C. 2010. Bovine tuberculosis: the genetic basis of host susceptibility. *Proc. Biol. Sci.* **277**: 2737–2745. [Medline] [CrossRef]
2. Ameni, G., Aseffa, A., Engers, H., Young, D., Gordon, S., Hewinson, G. and Vordermeier, M. 2007. High prevalence and increased severity of pathology of bovine tuberculosis in Holsteins compared to zebu breeds under field cattle husbandry in central Ethiopia. *Clin. Vaccine Immunol.* **14**: 1356–1361. [Medline] [CrossRef]
3. Bermingham, M. L., More, S. J., Good, M., Cromie, A. R., Hig-

gins, I. M., Brotherstone, S. and Berry, D. P. 2009. Genetics of tuberculosis in Irish Holstein-Friesian dairy herds. *J. Dairy Sci.* **92**: 3447–3456. [Medline] [CrossRef]

4. Brotherstone, S., White, I. M. S., Coffey, M., Downs, S. H., Mitchell, A. P., Clifton-Hadley, R. S., More, S. J., Good, M. and Woolliams, J. A. 2010. Evidence of genetic resistance of cattle to infection with *Mycobacterium bovis*. *J. Dairy Sci.* **93**: 1234–1242. [Medline] [CrossRef]
5. Burgner, D., Usen, S., Rockett, K., Jallow, M., Ackerman, H., Cervino, A., Pinder, M. and Kwiatkowski, D. P. 2003. Nucleotide and haplotypic diversity of the NOS2A promoter region and its relationship to cerebral malaria. *Hum. Genet.* **112**: 379–386. [Medline]
6. Castillo-Velázquez, U., Gomez-Flores, R., Tamez-Guerra, R., Tamez-Guerra, P. and Rodríguez-Padilla, C. 2013. Differential responses of macrophages from bovines naturally resistant or susceptible to *Mycobacterium bovis* after classical and alternative activation. *Vet. Immunol. Immunopathol.* **154**: 8–16. [Medline] [CrossRef]
7. Chan, J., Tanaka, K., Carroll, D., Flynn, J. and Bloom, B. R. 1995. Effects of nitric oxide synthase inhibitors on murine infection with *Mycobacterium tuberculosis*. *Infect. Immun.* **63**: 736–740. [Medline]
8. Evans, J. T., Smith, E. G., Banerjee, A., Smith, R. M., Dale, J., Innes, J. A., Hunt, D., Tweddell, A., Wood, A., Anderson, C., Hewinson, R. G., Smith, N. H., Hawkey, P. M. and Sonnenberg, P. 2007. Cluster of human tuberculosis caused by *Mycobacterium bovis*: evidence for person-to-person transmission in the UK. *Lancet* **369**: 1270–1276. [Medline] [CrossRef]
9. Gómez, L. M., Anaya, J. M., Vilchez, J. R., Cadena, J., Hinojosa, R., Vélez, L., Lopez-Nevot, M. A. and Martín, J. 2007. A polymorphism in the inducible nitric oxide synthase gene is associated with tuberculosis. *Tuberculosis (Edinb.)* **87**: 288–294. [Medline] [CrossRef]
10. Hermeyer, K., Jacobsen, B., Spergser, J., Rosengarten, R. and Hewicker-Trautwein, M. 2011. Detection of *Mycoplasma bovis* by in-situ hybridization and expression of inducible nitric oxide synthase, nitrotyrosine and manganese superoxide dismutase in the lungs of experimentally-infected calves. *J. Comp. Pathol.* **145**: 240–250. [Medline] [CrossRef]
11. Hsu, Y. H., Chen, C. W., Sun, H. S., Jou, R., Lee, J. J. and Su, I. J. 2006. Association of *NRAMP 1* gene polymorphism with susceptibility to tuberculosis in Taiwanese aboriginals. *J. Formos. Med. Assoc.* **105**: 363–369. [Medline] [CrossRef]
12. Janowski, T., Barański, W., Lukasik, K., Skarzyński, D., Rudowska, M. and Zduńczyk, S. 2013. Prevalence of subclinical endometritis in repeat breeding cows and mRNA expression of tumor necrosis factor alpha and inducible nitric oxide synthase in the endometrium of repeat breeding cows with and without subclinical endometritis. *Pol. J. Vet. Sci.* **16**: 693–699. [Medline]
13. Jiang, Y. N. 2002. Statics yearbook of animal and plant health inspection and quarantine, Republic of China.
14. Kadarmideen, H. N., Ali, A. A., Thomson, P. C., Müller, B. and Zinsstag, J. 2011. Polymorphisms of the *SLC11A1* gene and resistance to bovine tuberculosis in African Zebu cattle. *Anim. Genet.* **42**: 656–658. [Medline] [CrossRef]
15. Kun, J. F., Mordmüller, B., Perkins, D. J., May, J., Mercereau-Puijalon, O., Alpers, M., Weinberg, J. B. and Kremsner, P. G. 2001. Nitric oxide synthase $2^{\text{Lambaréné}}$ (G-954C), increased nitric oxide production, and protection against malaria. *J. Infect. Dis.* **184**: 330–336. [Medline] [CrossRef]
16. Leandro, A. C. C. S., Rocha, M. A. and Lamoglia-Souza, A. VandeBerg. J. L., Rolla, V. C. and Bonecini-Almeida, M. G. 2013. No Association of IFNG+874T/A SNP and NOS2A-954G/C SNP Variants with Nitric Oxide Radical Serum Levels or Susceptibility to Tuberculosis in a Brazilian Population Subset. *Biomed. Res. Int.* doi: 10.1155/2013/901740.
17. Levesque, M. C., Hobbs, M. R., O'Loughlin, C. W., Chancellor, J. A., Chen, Y., Tkachuk, A. N., Booth, J., Patch, K. B., Allgood, S., Pole, A. R., Fernandez, C. A., Mwaikambo, E. D., Mutabingwa, T. K., Fried, M., Sorensen, B., Duffy, P. E., Granger, D. L., Anstey, N. M. and Weinberg, J. B. 2010. Malaria severity and human nitric oxide synthase type 2 (NOS2) promoter haplotypes. *Hum. Genet.* **127**: 163–182. [Medline] [CrossRef]
18. Liu, W., Cao, W. C., Zhang, C. Y., Tian, L., Wu, X. M., Habbema, J. D., Zhao, Q. M., Zhang, P. H., Xin, Z. T., Li, C. Z. and Yang, H. 2004. *VDR* and *NRAMP1* gene polymorphisms in susceptibility to pulmonary tuberculosis among the Chinese Han population: a case-control study. *Int. J. Tuberc. Lung Dis.* **8**: 428–434. [Medline]
19. Ma, M. J., Xie, L. P., Wu, S. C., Tang, F., Li, H., Zhang, Z. S., Yang, H., Chen, S. L., Liu, N., Liu, W. and Cao, W. C. 2010. Toll-like receptors, tumor necrosis factor-α, and interleukin-10 gene polymorphisms in risk of pulmonary tuberculosis and disease severity. *Hum. Immunol.* **71**: 1005–1010. [Medline] [CrossRef]
20. MacMicking, J. D., North, R. J., LaCourse, R., Mudgett, J. S., Shah, S. K. and Nathan, C. F. 1997. Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proc. Natl. Acad. Sci. U.S.A.* **94**: 5243–5248. [Medline] [CrossRef]
21. Möller, M., Nebel, A., Valentonyte, R., van Helden, P. D., Schreiber, S. and Hoal, E. G. 2009. Investigation of chromosome 17 candidate genes in susceptibility to TB in a South African population. *Tuberculosis (Edinb.)* **89**: 189–194. [Medline] [CrossRef]
22. Nicholson, S., Bonecini-Almeida, M. G., Lapa e Silva, J. R., Nathan, C., Xie, Q. W., Mumford, R., Weidner, J. R., Calaycay, J., Geng, J., Boechat, N., Linhares, C., Rom, W. and Ho, J. L. 1996. Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *J. Exp. Med.* **183**: 2293–2302. [Medline] [CrossRef]
23. Paixão, T. A., Ferreira, C., Borges, A. M., Oliveira, D. A., Lage, A. P. and Santos, R. L. 2006. Frequency of bovine *Nramp1* (*Slc11a1*) alleles in Holstein and Zebu breeds. *Vet. Immunol. Immunopathol.* **109**: 37–42. [Medline] [CrossRef]
24. Pascual, M., López-Nevot, M. A., Cáliz, R., Koeleman, B. P., Balsa, A., Pascual-Salcedo, D. and Martín, J. 2002. Genetic determinants of rheumatoid arthritis: the inducible nitric oxide synthase (NOS2) gene promoter polymorphism. *Genes Immun.* **3**: 299–301. [Medline] [CrossRef]
25. Pereira-Suárez, A. L., Estrada-Chávez, C., Arriaga-Díaz, C., Espinosa-Cueto, P. and Mancilla, R. 2006. Coexpression of NRAMP1, iNOS, and nitrotyrosine in bovine tuberculosis. *Vet. Pathol.* **43**: 709–717. [Medline] [CrossRef]
26. Reséndiz-Hernández, J. M., Sansores, R. H., Hernández-Zenteno, R. J., Vargas-Alarcón, G., Colín-Barenque, L., Velázquez-Uncal, M., Camarena, A., Ramírez-Venegas, A. and Falfán-Valencia, R. 2015. Identification of genetic variants in the *TNF* promoter associated with COPD secondary to tobacco smoking and its severity. *Int. J. Chron. Obstruct. Pulmon. Dis.* **10**: 1241–1251. [Medline]
27. le Roex, N., Koets, A. P., van Helden, P. D. and Hoal, E. G. 2013. Gene polymorphisms in African buffalo associated with susceptibility to bovine tuberculosis infection. *PLoS ONE* **8**: e64494. [Medline] [CrossRef]
28. Qidwai, T. and Jamal, F. 2010. Inducible nitric oxide synthase (*iNOS*) gene polymorphism and disease prevalence. *Scand. J.*

Immunol. **72**: 375–387. [Medline] [CrossRef]
29. Qu, Y., Tang, Y., Cao, D., Wu, F., Liu, J., Lu, G., Zhang, Z. and Xia, Z. 2007. Genetic polymorphisms in alveolar macrophage response-related genes, and risk of silicosis and pulmonary tuberculosis in Chinese iron miners. *Int. J. Hyg. Environ. Health* **210**: 679–689. [Medline] [CrossRef]
30. Schiller, I., Vordermeier, H. M., Waters, W. R., Whelan, A. O., Coad, M., Gormley, E., Buddle, B. M., Palmer, M., Thacker, T., McNair, J., Welsh, M., Hewinson, R. G. and Oesch, B. 2010. Bovine tuberculosis: effect of the tuberculin skin test on *in vitro* interferon gamma responses. *Vet. Immunol. Immunopathol.* **136**: 1–11. [Medline] [CrossRef]
31. Selvaraj, P., Sriram, U., Mathan Kurian, S., Reetha, A.M. and Narayanan, P.R. 2001. Tumour necrosis factor alpha (−238 and −308) and beta gene polymorphisms in pulmonary tuberculosis: haplotype analysis with *HLA-A, B* and *DR* genes. *Tuberculosis* **81** (5e6) 335e341.
32. Shu, D., Heiser, A., Wedlock, D. N., Luo, D., de Lisle, G. W. and Buddle, B. M. 2014. Comparison of gene expression of immune mediators in lung and pulmonary lymph node granulomas from cattle experimentally infected with *Mycobacterium bovis*. *Vet. Immunol. Immunopathol.* **160**: 81–89. [Medline] [CrossRef]
33. Sun, L., Song, Y., Riaz, H., Yang, H., Hua, G., Guo, A. and Yang, L. 2012. Polymorphisms in toll-like receptor 1 and 9 genes and their association with tuberculosis susceptibility in Chinese Holstein cattle. *Vet. Immunol. Immunopathol.* **147**: 195–201. [Medline] [CrossRef]
34. Trovoada, M. J., Martins, M., Ben Mansour, R., Sambo, M. R., Fernandes, A. B., Antunes Gonçalves, L., Borja, A., Moya, R., Almeida, P., Costa, J., Marques, I., Macedo, M. P., Coutinho, A., Narum, D. L. and Penha-Gonçalves, C. 2014. NOS2 variants reveal a dual genetic control of nitric oxide levels, susceptibility to Plasmodium infection, and cerebral malaria. *Infect. Immun.* **82**: 1287–1295. [Medline] [CrossRef]
35. Velez, D. R., Hulme, W. F., Myers, J. L., Weinberg, J. B., Levesque, M. C., Stryjewski, M. E., Abbate, E., Estevan, R., Patillo, S. G., Gilbert, J. R., Hamilton, C. D. and Scott, W. K. 2009. NOS2A, TLR4, and IFNGR1 interactions influence pulmonary tuberculosis susceptibility in African-Americans. *Hum. Genet.* **126**: 643–653. [Medline] [CrossRef]
36. Widdison, S., Ashley, G. R., Howard, C. J. and Coffey, T. J. 2007. Characterisation of bovine inducible nitric oxide synthase. *Vet. Immunol. Immunopathol.* **117**: 302–309. [Medline] [CrossRef]