

RESEARCH ARTICLE

Association of NOS2A gene polymorphisms with susceptibility to bovine tuberculosis in Chinese Holstein cattle

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Abstract

Bovine tuberculosis (bTB) is a global zoonotic disease that has detrimental economic impacts worldwide. The *NOS2A* gene plays a key role in immunological control of many infectious diseases. However, research on the association between *NOS2A* polymorphisms and bTB infection in Holstein cattle reared on the Yunnan-Guizhou plateau of China is scarce. This study investigated a possible linkage between *NOS2A* polymorphisms and risk of developing bTB in Chinese Holstein cattle. The *NOS2A* gene was genotyped in 144 bTB-infected Holstein cows and 139 healthy controls were genotyped through nucleotide sequencing. Ten single-nucleotide polymorphisms (SNPs) were detected, six of which were associated with susceptibility/resistance patterns of bTB. Furthermore, the C/T genotypes of 671 and 2793, and T/T genotype of E22 (+15) were significantly associated with susceptibility risk; the G/A genotype of 2857, T/T genotype of E9 (+65), and C/C genotype of E9 (+114) probably increased resistance to bTB. In addition, the haplotypes of *NOS2A-2* and *NOS2A-9* were risk factors for bTB susceptibility, while the *NOS2A-5* and *NOS2A-8* haplotypes were contributing protective variants against tuberculosis. There is a significant association between variation in SNPs of *NOS2A* and tuberculosis susceptibility/resistance pattern. These findings suggest that substitution of genetic selection would be helpful for eradicating bTB. However, further investigation is required to study the underlying mechanism through which *NOS2A* polymorphisms affect bTB infection.

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Introduction

Bovine tuberculosis (bTB) is a chronic infectious disease of great concern in cows. The disease is caused by the *Mycobacterium tuberculosis* pathogen. bTB has been documented in many countries, including England, Ireland, Brazil, and China [1–4], where it not only causes large financial losses to the cattle industry but also poses a threat to the health of human beings and wild animals [5]. In developed countries, the prevalence of human tuberculosis (TB) is

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Abbreviations: AIC, Akaike's information criterion; bTB, Bovine Tuberculosis; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NO, Nitric oxide; NOS, NO synthase; PIC, Polymorphism information content; SNP, Single nucleotide polymorphism; TB, Tuberculosis; TST, Tuberculin skin test.

relatively low, while it remains a potential hazard with an incidence of 10–15% in developing countries due to the high prevalence of bTB [6]. It has been reported that one-third of humans are infected with bTB around the world and 10% of these infected people will develop active TB, suggesting that individual differences may play a critical role in the process of TB infection [7,8].

Nitric oxide (NO) plays a vital role in many physiological and pathological processes, and it is synthesized from the amino acid L-arginine by NO synthase (NOS). NOS is found in three distinct isoforms: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) [9,10]. iNOS is encoded by the *NOS2A* gene, which is under the transcriptional control of inflammatory mediators produced by immunocompetent cells such as macrophages and neutrophils [11]. iNOS-deficient mice are highly susceptible to TB infection, characterized by atypical granulomas that can facilitate mycobacterial reactivation, dissemination, and transmission [12,13]. Previous studies suggest that the production or activity of iNOS may be influenced by several single nucleotide polymorphisms (SNPs) within the *NOS2A* gene [10,14]. The rs8078340 SNP causes a nucleotide change from guanine to adenine, and this change decreases the amount of bound DNA–protein complex, leading to nitric oxide reduction [15]. Two SNPs (rs1800482, a cytosine to guanine nucleotide change, and rs9282799, a guanine to adenine nucleotide change) in promoter mutations of the *NOS2* gene are associated with a high baseline level of iNOS activity, which increases the synthesis of nitric oxide [16]. The rs57234985 (a thymine to cytosine nucleotide change) SNP is associated with low levels of exhaled nitric oxide [15,17].

Nutritional, environmental and genetic factors are involved in the pathogenesis of TB, and multiple genes control it. Different diseases can be tracked by SNP analysis [18–21]. It has been documented that the rs2779249 and rs2301369 SNPs are associated with TB in Brazilians, and rs2779249, rs9282799, and rs8078340 are associated with the diseased in South African populations [22]. The rs2297518 SNP in exon 16 may act as an important protection factor against development of pulmonary TB in Chinese miners [23]. Therefore, the identification of genetic traits that influence susceptibility or resistance to bTB might offer alternative methods of controlling the disease through genetic selection [24]. However, data regarding the association between *NOS2A* polymorphisms and susceptibility to bTB in Holstein cattle reared on the Yunnan–Guizhou plateau of China are scant. Therefore, we characterized the diversity and SNP variations in the *NOS2A* gene of 144 bTB-infected cattle and 139 healthy controls to help explain the individual differences in susceptibility to bTB.

Materials and methods

Ethics

All of the experiments were conducted according to the rules and regulations of the Administration of Affairs Concerning Experimental Animals published by the Ministry of Science and Technology, China, in 2008. All animal experiments were conducted according to the Chinese Guidelines for Institutional Animal Care and Use Committee of the Yunnan Agricultural University (Approval number: IACUC-20132030301).

Sample collection and TB detection

The dairy cows used for this research were reared on high-altitude (more than 1500 m) farms located in Kunming, Dali, and Yuxi. The samples were collected according to a report of the general investigation of TB in dairy cows by Yunnan Provincial Animal Disease Prevention and Control Center, Dali Animal Disease Prevention and Control Center and Yuxi Animal Disease Prevention and Control Center. A total of 283 Chinese Holstein cows including 144

bTB-positive and 139 bTB-negative (controls) were studied. Blood samples from each experimental cow were collected into a disposable heparin sodium anticoagulation vacutainer.

Genomic DNA extraction

Genomic DNA was extracted from blood using a Whole Blood Genomic DNA Extraction Kit (Magen Bio, Guangzhou, China) following the manufacturer's instructions. Samples were subsequently diluted at a concentration of 10 $\mu\text{mol/L}$ and stored at -20°C until use. DNA quality was identified via agarose gel electrophoresis.

Primer design and synthesis

The primers were designed using Primer Premier 6 software according to the sequence of NOS2A (AC_000176.1), published in the National Center for Biotechnology, and synthesized by Takara Company. Information on primers is given in [Table 1](#).

PCR amplification conditions for NOS2A

Samples for PCR were prepared in a volume of 50 μL containing 4.0 μL dNTPs mixture (2.5 mmol/L), 5.0 μL 10 \times buffer, 0.5 μL Taq polymerase, 1.0 μL each primer (10 mmol/L), 5.0 μL DNA, and 34.5 μL RNase-free water. PCR conditions were as follows: pre-denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, with a final elongation at 72°C for 8 min.

DNA sequencing and detection of polymorphisms

To analyze the PCR amplification products a 1% agarose gel electrophoresis. Products after staining that showed a bright band without nonspecific amplification were sent to Suke Biotechnology (Kunming, China) for sequencing. DNASTar Lasergene 11.1 (Madison, WI, USA) was used to compare the sequences with the whole genome of NOS2A.

Statistical analysis

The χ^2 test ($\alpha = 0.05$, $P > 0.05$) was used to determine Hardy-Weinberg equilibrium (HWE) and compare genotype distribution and allele frequency between bTB-infected cattle and controls. Polymorphism information content (PIC) was assessed as follows: $\text{PIC} > 0.5$ (high polymorphism), $0.25 < \text{PIC} < 0.5$ (moderate polymorphism), and $\text{PIC} < 0.25$ (low polymorphism).

Table 1. Primer sequences for the NOS2A gene.

Primer	Sequence (5'-3')	Length	Location	Products (bp)	Annealing temperature ($^{\circ}\text{C}$)
NOS2A-F1	CTTCTTCCTTGCCCGTTTCTA	22	Intron1	898	59.6
NOS2A-R1	GAGGGTGGTGTGGAAC TAACA	21	Intron2		
NOS2A-F2	GCAGGGGTTTGGTTCAGTTAG	21	Intron5	1004	60.0
NOS2A-R2	CCAGCCTAAAGCCTGCTACATA	22	Intron7		
NOS2A-F3	CTCCAGGTGGGATGAACTTAGA	22	Intron7	932	59.6
NOS2A-R3	TAGTTGCTGCTTAGGGGGAGTA	22	Intron9		
NOS2A-F4	AGCAAACTGGGCTGTGTTCTA	22	Intron17	904	59.0
NOS2A-R4	AGCCCCGGTATGTAGGTATGA	22	Intron18		
NOS2A-F5	GGTGAGGTGATGGTGAATGTAG	22	Intron21	908	59.6
NOS2A-R5	GAGGTGAAGGCTCACAGAAGTAA	23	Intron23		

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The SNPstats software (<https://www.snpstats.net>) and SPSS v19.0 program (SPSS, Chicago, IL) were used for statistical analysis.

Results

Analysis of polymorphisms in the bovine *NOS2A* gene

As shown in [Table 2](#), 10 SNPs were identified in the *NOS2A* gene.

Genotype distribution and allele frequency of SNPs in bTB-infected and non-infected cattle

The genotypic and allelic frequencies of all 10 SNPs, except for E9 (+65) in the control group, exceeded up to 5% both in both groups, suggesting that Hardy–Weinberg equilibrium was satisfied ($P > 0.05$). There were two genotypes at loci 140, 671, E18 (-292), E18 (-133), 2243, 2793, and 2857, and three at E9 (+65), E9 (+114), and E22 (+15). PIC values were less than 0.25, indicating low polymorphism in six SNPs ([Table 3](#)).

Association analysis between SNP and bTB susceptibility

Logistic regression was used to investigate the association between single loci of SNP and bTB susceptibility. As shown in [Table 4](#), the SNPs of 671, 2793, E9 (+65), E9 (+114), 2857, and E22 (+15) were significantly associated with susceptibility or resistance to bTB; We also calculated odds ratios and 95% confidence intervals (CIs) ([Table 4](#)), and found that the C/T genotype frequencies of both 671 and 2793 were higher in bTB-infected cattle than in controls, indicating that the C/T genotype of these SNPs might be associated with higher susceptibility to bTB. The G/A genotype of 2857 was only found in the control group; therefore, it might confer resistance to bTB.

To explore potential genotypic risk factors for TB, we analyzed genetic models using Akaike's information criterion We found that locus E9 (+65) showed recessive inheritance and the T/T genotype of E9 (+65) was only found in the control group, suggesting that these genotypes are probably associated with resistance to bTB. The best genetic model of locus E9 (+114) was log-additive inheritance, and the C/C genotype frequency of E9 (+114) in bTB-infected cattle (64.0%) was lower than control (86.0%), suggesting that these genotypes were protective against TB. The best genetic model for locus E22 (+15) was log-additive inheritance, and the T/T genotype frequency of E22 (+15) in bTB-infected cattle (92.0%) was higher than that of controls (81.0%) as shown in [Table 4](#).

Table 2. SNP sites and change in amino acid.

Primer	Site	Variation	Location	Change in amino acid
F1/R1	140	C/G	Exon-2	--
F2/R2	671	C/T	Exon-6	--
F3/R3	E9 (+65)	C/T	Intron-9	--
	E9 (+114)	T/C	Intron-9	--
F4/R4	E18 (-292)	C/T	Intron-17	--
	E18 (-133)	G/A	Intron-17	--
	2243	G/A	Exon-18	--
F5/R5	2793	C/T	Exon-22	--
	2857	G/A	Exon-22	A-T
	E22 (+15)	T/C	Intron-22	--

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Table 3. Distribution of 10 polymorphic genotypes and allele frequency in bTB-infected cattle and controls.

SNPs	Group	Genotype frequency			Allele frequency		PIC	χ^2	HWE P
		C/C	C/G	G/G	C	G			
140	Infected cattle	135 (0.94)	9 (0.06)	0 (0)	279 (0.97)	9 (0.03)	0.057	0.15	1
	Non-infected cattle	133 (0.96)	6 (0.04)	0 (0)	272 (0.98)	6 (0.02)			
671	Infected cattle	126 (0.88)	18 (0.12)	0 (0)	270 (0.94)	18 (0.06)	0.074	0.64	1
	Non-infected cattle	132 (0.95)	7 (0.05)	0 (0)	271 (0.95)	7 (0.03)			
E9(+65)	Infected cattle	115 (0.8)	29 (0.2)	0 (0)	259 (0.9)	29 (0.1)	0.177	1.81	0.36
	Non-infected cattle	112 (0.81)	22 (0.16)	5 (0.04)	246 (0.88)	32 (0.12)			
E9(+114)	Infected cattle	92 (0.64)	47 (0.33)	5 (0.03)	231 (0.8)	57 (0.2)	0.212	0.11	1
	Non-infected cattle	119 (0.86)	20 (0.14)	0 (0)	258 (0.93)	20 (0.07)			
E18(-292)	Infected cattle	127 (0.88)	17 (0.12)	0 (0)	271 (0.94)	17 (0.06)	0.106	0.57	1
	Non-infected cattle	124 (0.89)	15 (0.11)	0 (0)	263 (0.95)	15 (0.05)			
E18(-133)	Infected cattle	120 (0.83)	24 (0.17)	0 (0)	264 (0.92)	24 (0.08)	0.136	1.19	0.6
	Non-infected cattle	119 (0.86)	20 (0.14)	0 (0)	258 (0.93)	20 (0.07)			
2243	Infected cattle	115 (0.8)	29 (0.2)	0 (0)	259 (0.9)	29 (0.1)	0.150	1.81	0.36
	Non-infected cattle	116 (0.83)	23 (0.17)	0 (0)	255 (0.92)	23 (0.08)			
2793	Infected cattle	118 (0.82)	26 (0.14)	0 (0)	262 (0.91)	26 (0.09)	0.122	1.41	0.6
	Non-infected cattle	126 (0.91)	13 (0.09)	0 (0)	265 (0.95)	13 (0.05)			
2857	Infected cattle	144 (1)	0 (0)	0 (0)	288 (1)	0 (0)	0.038	0.39	1
	Non-infected cattle	125 (0.9)	14 (0.1)	0 (0)	264 (0.95)	14 (0.05)			
E22(+15)	Infected cattle	132 (0.92)	12(0.08)	0 (0.00)	276 (0.96)	12 (0.04)	0.122	0.27	1
	Non-infected cattle	112 (0.81)	25 (0.18)	2 (0.01)	249 (0.9)	29 (0.1)			

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Haplotype analysis

We identified and analyzed haplotypes based on *NOS2A* gene polymorphism and discarded total occurrence frequencies below 0.01. *NOS2A-2*, *NOS2A-5*, *NOS2A-8*, and *NOS2A-9* had significant associations with bTB susceptibility/resistance ($P < 0.05$). Further analysis revealed that the frequency of haplotype *NOS2A-2* (OR, 3.97; 95% CI, 1.85–8.55) was significantly higher in bTB-infected cattle ($P = 0.1098$) than in controls ($P = 0.0479$), and that of *NOS2A-9* was only found in bTB-infected cattle ($P = 0.1098$), suggesting that these two haplotypes are risk factors for bTB. In contrast, the haplotype frequency of *NOS2A-5* (OR, 0.24; 95% CI, 0.24) was significantly higher in controls ($P = 0.0577$) and that of *NOS2A-8* was only found in controls, indicating that these two haplotypes are protective factors against TB (Table 5).

Discussion

Polymorphism in the *NOS2A* gene is associated with many diseases, including cerebral palsy, malaria, type 2 diabetes mellitus, asthma, rheumatoid arthritis, purpura, and coronary heart disease [25–32]. In addition, recent findings have demonstrated that the SNP locus

Table 4. Association analysis between SNP sites and tuberculosis infection in cattle.

SNPs	Models	Genotype	bTB-infected cattle	Non-infected cattle	OR (95%CI)	P-value	AIC
140	---	C/C	135 (93.8%)	133 (95.7%)	1.00	0.47	395.7
		C/G	9 (6.2%)	6 (4.3%)	1.48 (0.51–4.27)		
671	---	C/C	126 (87.5%)	132 (95%)	1.00	0.024	391.2
		C/T	18 (12.5%)	7 (5%)	2.69 (1.09–6.67)		
E9(+65)	Recessive	C/C-C/T	144 (100%)	134 (96.4%)	1.00	0.0073	389
		T/T	0 (0%)	5 (3.6%)	NA		
E9(+114)	Log-additive	---	---	---	3.32 (1.90–5.80)	<0.0001	375.9
E18(-292)	---	C/C	127 (88.2%)	124 (89.2%)	1.00	0.79	396.2
		C/T	17 (11.8%)	15 (10.8%)	1.11 (0.53–2.31)		
E18(-133)	---	G/G	120 (83.3%)	119 (85.6%)	1.00	0.6	396
		G/A	24 (16.7%)	20 (14.4%)	1.19 (0.62–2.27)		
2243	---	G/G	115 (79.9%)	116 (83.5%)	1.00	0.43	395.6
		G/A	29 (20.1%)	23 (16.6%)	1.27 (0.69–2.33)		
2793	---	C/C	118 (81.9%)	126 (90.7%)	1.00	0.032	391.6
		C/T	26 (18.1%)	13 (9.3%)	2.14 (1.05–4.35)		
2857	---	G/G	144 (100%)	125 (89.9%)	1.00	<0.0001	375.6
		G/A	0 (0%)	14 (10.1%)	NA		
E22(+15)	Log-additive	---	---	---	0.38 (0.19–0.76)	0.0039	387.9

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polymorphism of the *NOS2A* gene as well as the polymorphism of the microsatellite promoter is associated with TB. Velez *et al.* found that the *NOS2A* and *TLR2* genes regulate epistatic interactions as modifiers of SLC11A1-mediated TB risk in African Americans and Caucasians [33]. Moller *et al.* reported that haplotypes composed of two SNP loci in the *NOS2A* gene promoter, rs9282799 and rs8078340, are significantly associated with TB in South Africans [22]. In addition, the *NOS2A* gene promoter region (CCTTT) has a significant protective effect against TB in various Indian populations [34]. A study African Americans found that multiple SNPs in the *NOS2A* gene were associated with TB, and some sites were also found to have synergistic effects on the *TLR4* and *IFNGR1* genes and affect TB risk [15].

The *NOS2A* gene regulates the NO levels by encoding inducible NOS (iNOS) and mediating immune responses to TB and other infectious diseases. iNOS is a pivotal regulator of susceptibility to tuberculosis infection in mice [35]. It has been documented that NO has the

Table 5. Association analysis between *NOS2A* haplotypes and bTB infection.

NO.	Haplotypes	Freq of bTB-infected cattle	Freq of non-infected cattle	Freq	OR (95%CI)	P-value
NOS2A-1	CCCCGGCGT	0.4977	0.5644	0.4836	1	--
NOS2A-2	CCCTCGGCGT	0.1098	0.0479	0.0923	3.97 (1.85–8.55)	0.0005
NOS2A-3	CCTCCGGCGT	0.0558	0.0532	0.0629	1.05 (0.48–2.29)	0.9
NOS2A-4	CCCCGACGT	0.0488	0.0315	0.0506	2.48 (0.88–6.97)	0.086
NOS2A-5	CCCCGGCGC	0.0059	0.0577	0.0461	0.24 (0.08–0.72)	0.012
NOS2A-6	CCCCGGTGT	0.043	0.0235	0.0429	2.51 (0.89–7.04)	0.083
NOS2A-7	CCCCAGCGT	0.0192	0.0264	0.0306	0.77 (0.23–2.61)	0.68
NOS2A-8	CCCCGGCAT	NA	0.0282	0.0239	0.08 (0.01–0.71)	0.024
NOS2A-9	CTCCGGCGT	0.0246	NA	0.023	0.00 (-Inf–Inf)	1
NOS2A-10	CCCCTGGCGT	0.0096	0.0144	0.0174	0.70 (0.12–3.96)	0.69

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direct cytotoxicity to mycobacteria [36,37]. Therefore, the *NOS2A* gene is associated with bTB susceptibility.

In the past decade, many studies have investigated the relationship between the *NOS2A* gene and human TB, but there is insufficient research on the correlation between *NOS2A* polymorphisms and bTB susceptibility, especially on the plateau of China. Therefore, we studied this topic in Holstein cattle reared on the Yunnan Plateau. We found that SNP 2857 caused an amino acid change from alanine to threonine, and that PIC values of 10 SNPs were less than 0.25, suggesting low polymorphism.

Furthermore, haplotypes *NOS2A*-2 and *NOS2A*-9 were found to be risk factors for bTB susceptibility, while the *NOS2A*-5 and *NOS2A*-8 were considered to protect against bTB. The occurrence and development of bTB is affected by various factors including pathogens, environmental causes, and susceptibility genes. Our results offer information that could improve breeding for bTB-resistant cattle, potentially eradicating the disease over time. Meanwhile, *NOS2A* polymorphisms could be potential biomarkers for predicting bTB risk. A multi-model deep learning framework based on convolutional neural networks, which has been verified for the prediction of risk in Alzheimer's disease and gastric cancer [38,39], may be used to aid in the development of the prediction model. Moreover, the exact mechanisms involved bTB susceptibility and resistance need to be verified in further experiments. For example, during TB infection, the gut microbiota is modulated and may be responding to immunological changes in the host, as the *NOS2A* gene is involved in the immunological regulation of many infectious diseases. Therefore, the relationship between *NOS2A* polymorphisms and the gut microbiome during bTB infection could be investigated further using a metagenomic analysis tool [40]. Meanwhile, mRNA modifications are potentially new insights into this biological basis of bTB susceptibility. The recent progress in N4-Acetylcytidine on RNA expression is also playing key role on a lot of diseases, and people have found that the *NOS2A* abnormal expression is mediated through mRNA N4-Acetylcytidine [41].

This study also has its limitation. Firstly, the *NOS2A* polymorphisms associated with bTB in this study were intend to be analyzed using meta-analysis, which has been reported in several publications [42–45], but the sample size limitation in this study prevented the application of meta-analysis. Secondly, causal effects of this gene's variants on the risk of bTB should be explored through integrating gene expression data under the Mendelian Randomization framework [46,47], to determine if the genetic variants may affect the bTB susceptibility through regulating the gene's expression level. Unfortunately, the expression data is not available.

Conclusion

The C/T genotypes of 671 and 2793 and T/T genotype of E22 (+15) may be associated with susceptibility to bTB; the G/A genotype of 2857, T/T genotype of E9 (+65), and C/C genotype of E9 (+114) probably increase resistance to bTB. In addition, the haplotypes of *NOS2A*-2 and *NOS2A*-9 may be risk factors for bTB, and those of *NOS2A*-5 and *NOS2A*-8 may be protective factors against bTB. However, the underlying causality and mechanisms are unclear; therefore, further investigation and confirmation are required.

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Author Contributions

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