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Toll-like receptor 2 gene polymorphisms in Chinese Holstein cattle and their associations with bovine tuberculosis

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ABSTRACT

This study evaluated whether there was an association between polymorphisms within the *Toll-like receptor 2* gene (*TLR2*) of Chinese Holstein cattle and susceptibility to bovine tuberculosis (BTB). In a case–control study including 210 BTB cases and 237 control cattle, we found only two common single-nucleotide polymorphisms (SNPs) within the entire coding region of the *TLR2* gene, A631G (rs95214857) and T1707C (rs1388116488). Additionally, the allele and genotype distributions of A631G and T1707C were not different between case and control groups, indicated that these SNPs were not associated with susceptibility to BTB. These results suggested that polymorphisms in the *TLR2* gene might not play a significant role in the BTB risk in Chinese Holstein cattle.

1. Introduction

Bovine tuberculosis (BTB) is a chronic infectious disease caused by *Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis* complex (le Roex et al., 2013). To date, because of the lack of effective preventive and control policies, BTB remains common all over the world and has caused severe economic losses in the cattle industry, especially in developing countries (Proano-Perez et al., 2011; Song et al., 2014; Wang et al., 2015). BTB is also a threat for public health, as *M. bovis* may affect cattle, humans, and all kinds of wildlife (Michel et al., 2006, 2009; le Roex et al., 2013)).

The clinical occurrence rate of BTB in different cattle breeds suggests that genetic factors may play a crucial role in susceptibility to BTB (Vordermeier et al., 2012; Alfano et al., 2014). Previous studies have identified several genetic factors which may enhance or reduce susceptibility to BTB and influence development of the disease (Acevedo-Whitehouse et al., 2005; Song et al., 2014). Therefore, selective breeding with BTB-resistant genetic markers might represent a new approach to supporting BTB control (Alfano et al., 2014).

The Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs), and play fundamental roles in pathogen recognition and activation of innate immunity. The TLRs include 13 members (TLRs 11-13), and different TLRs can recognize different antigens from diverse pathogens (Beutler, 2004; Dasari et al., 2008). Single-nucleotide polymorphisms (SNPs) in the *Toll-like receptor* (*TLR*) genes have been reported to associate with susceptibility to tuberculosis indifferent animal species as well as humans (Alfano et al., 2014). To date, variants in *TLR1*, *TLR2*, *TLR4*, *TLR6* and *TLR9* have been associated with susceptibility to tuberculosis in humans (Ma et al., 2010; Selvaraj et al., 2010; Xue et al., 2010); additionally, variants in *TLR2*, *TLR4* and *TLR9* are associated with susceptibility to BTB in water buffalo (Alfano et al., 2014), and variants in *TLR1* and *TLR6*, but not in *TLR9*, have been shown to be associated with susceptibility to BTB in Chinese Holstein cattle (Song et al., 2014; Sun et al., 2012). However, these results seem to be quite diverse.

TLR2 is mainly localized on immune cells and respiratory epithelial cells, and recognize mycobacterial membrane components, including *Mycobacterium* (Akira et al., 2001). Then the TLR2 and membrane component complex connect adaptor molecules such as MyD88 and TRIF and activate MyD88-dependent and TRIF-dependent signaling pathways in macrophages or other cells. These responses lead to the secretion of inflammatory cytokines, chemokines, interferon and antimicrobial peptides (Kawai and Akira, 2010), resulting in the direct killing of the infected mycobacterial pathogens and the induction of adaptive immunity (Saiga et al., 2011). TLR2 is reported to be able to initiate the host defense

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against tuberculosis, especially in chronic infection (Kleinnijenhuis et al., 2011; Gopalakrishnan and Salgame, 2016). The objective of this study was to identify SNPs in the *TLR2* gene of Chinese Holstein cattle and assess their possible associations with BTB.

2. Materials and methods

2.1. Sample collection

In total, 447 Chinese Holstein cows (210 BTB-positive cases and 237 negative cases) were included in this study. These cows were 2–5 years of age, living in the same geographical region (northern China), with the same environmental and nutritional conditions. BTB was tested using both the comparative cervical tuberculin (CCT) test according to the People's Republic of China's standards for diagnosing animal TB (GB/T 186452002) and the interferon gamma (IFN-γ) assay to avoid false-positive or false-negative results. All of the procedures and tests were performed as previously described (Song et al., 2014). A cow was considered to be positive for BTB if positive results were observed for both tests and if the cow showed symptoms of emaciation, cough, dyspnea and lymphadenectasis. A cow was considered negative only if negative results were observed for both tests and the cow did not show clinical symptoms. Cows were divided into naturally infected and healthy groups according to results of these two tests. Out of 447 cows, 210 were positive (cases) and 237 were negative (controls). All procedures carried out on animals were approved by the Animal Care and Use Committee of the Henan University of Science and Technology.

Blood samples were collected from the cows and stored in tubes containing 1.5% EDTA-2K. Genomic DNA was extracted with the Blood Genomic DNA Extraction Kit (TaKaRa Bio, Dalian, China) and stored at −20 °C.

2.2. Genotyping

Polymorphisms within the *TLR2* gene were detected by PCR followed by direct sequencing. Four pairs of primers covering the complete *TLR2* coding region (2399 bp) were designed using primer 5.0 software based on the previously published sequence of the bovine *TLR2* gene (GenBank accession: AC-000174.1) (Table 1). The PCR reaction volume was 25 μl, which included 12.5 μl PCR Master Mix (Sangon Biotech, Shanghai, China), 1 μl of each primer, 1 μl genomic DNA, and 9.5 μl ultrapure water. The PCR reactions were performed as follows: an initial denaturation step at 94 °C for 10 min; 30 cycles of 94 °C for 30 s, 57–59 °C for 30 s, 72 °C for 50 s, and 72 °C for 10 min. PCR products were analyzed using 1% agarose gel electrophoresis and visualized after staining with ethidium bromide. The positive products were sent for bidirectional single pass sequencing analysis at Sangon Biotech. The genotyping failure rate was 1.34% for rs95214857 and 0% for rs1388116488.

2.3. Statistical analysis

Allele and genotype frequencies of the two SNPs were compared between cases and controls using the χ^2 test. Odds ratios and 95% confidence intervals were also calculated. The Hardy–Weinberg equilibrium (HWE) was assessed using a χ^2 test in each control group. Differences were considered statistically significant at $P < 0.05$. SPSS 17.0 (SPSS Inc., Chicago, IL, USA) software was used for the statistical analysis.

3. Results

Only two SNPs, A631G (rs95214857) and T1707C (rs1388116488) were found in the entire coding region of the *TLR2* gene (Fig. 1). The genotypes of these SNPs in control individuals both displayed Hardy–Weinberg equilibrium ($P > 0.05$). The G allele of A631G and the C allele of T1707C were not significantly different between cases and controls, and not associated with BTB ($P = 0.79$, OR = 0.96, 95% CI = 0.69–1.33; $P = 0.66$, OR = 1.08, 95% CI = 0.76–1.54, respectively) (Table 2). Additionally, the GA genotype of A631G and the CT genotype of T1707C were not significantly different between cases and controls, and not associated with BTB ($P = 0.71$, OR = 0.93, 95% CI = 0.63–1.36; $P = 0.57$, OR = 1.12, 95% CI = 0.76–1.67, respectively) (Table 2).

4. Discussion

To date, hundreds of SNPs have been identified in TLR-family genes. Some of these SNPs have been reported to associate with susceptibility to not only tuberculosis (Kleinnijenhuis et al., 2011), but also with *Helicobacter pylori* infection (Ravishankar Ram et al., 2015) and vitiligo (Traks et al., 2015). Among the TLRs, TLR2 is believed to be important in the initiation of the innate host defense against tuberculosis (Kleinnijenhuis et al., 2011). TLR2 also has a janus function in vivo as mediator of the role of bacterial products in balancing pro- and anti-inflammatory immune responses in *M. tuberculosis* infection (Piermattei et al., 2016). Since TLR2 played fundamental roles in pathogen recognition and activation of innate immunity, gene variations in the *TLR2* gene region might results in a significant change in the ability of immune cells to respond to *M. bovis* infection.

Some functional and genetic studies have concluded that SNPs or other gene variations in the *TLR2* gene region have the ability to modify gene expression level or alter the immune response to PAMPs (Lorenz et al., 2000; Merx et al., 2007; Mrabet-Dahbi et al., 2008). *TLR2* knockout mice exhibited enhanced susceptibility to infection with *M. tuberculosis* compared with wild-type mice and developed chronic lethal pneumonia (Kleinnijenhuis et al., 2011). Additionally, studies have indicated that some SNPs in the *TLR2* gene might be associated with susceptibility to tuberculosis in humans and in water buffalo (Song et al., 2014; Alfano et al., 2014).

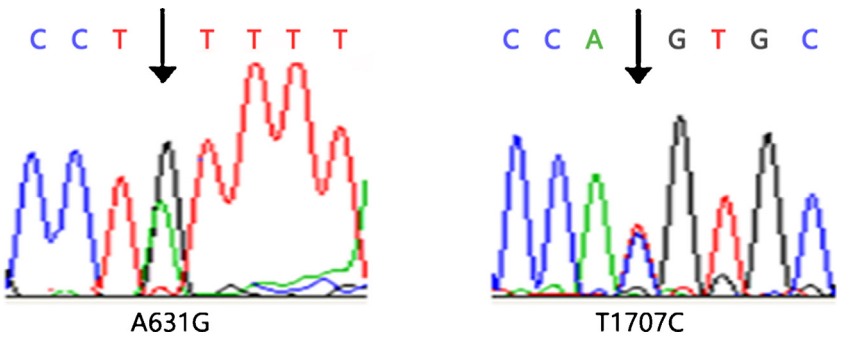

Fig. 1. SNPs sites in the *TLR2* gene sequencing of Chinese Holstein cattle. Black arrow: Heterozygous genotypes of A631G and T1707C sites.