Association study of Single Nucleotide Polymorphisms (SNP) in Toll-like Receptor 9 gene with bovine tuberculosis

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ABSTRACT
Toll-like Receptor 9 (TLR9) play an important role in recognition of components of intracellular pathogens like Mycobacterium bovis and subsequent activation of both innate and adaptive immune response and is potential strong candidates for investigating genetic basis of disease resistance. Present investigation was aimed at exploring the association of four SNPs in TLR9 gene with susceptibility/resistance against bovine tuberculosis infection in cattle. Three of SNPs under investigation (rs210982793, rs207807011, rs209190268) revealed polymorphism whereas monomorphism was observed in SNP rs55617140. SNP loci rs210982793 and rs207807011 were significantly (P < 0.01) associated with susceptibility to bovine tuberculosis in the case control population. Both these SNPs loci were non-synonymous, thus suggestive of their functional role in the immune response against bovine tuberculosis.

Key words: Bovine tuberculosis, Immune response, Resistance, SNP, Toll-like receptors.

INTRODUCTION
Bovine Tuberculosis (bTB) is a chronic disease of cattle caused by Mycobacterium bovis that typically infects the respiratory system and associated lymph nodes. bTB has serious consequences for transfer of animals, constrains in international trade of animals and their products, public health, biodiversity and farm profits (Neill et al., 1994, Dhama et al., 2013, Rodriguez-Campos et al., 2014). It costs an estimated $3 billion annually in global agricultural losses (Garnier et al., 2003) and is the fourth most important livestock disease worldwide (Perry et al., 2013). A typical feature of Mycobacterium bovis infection is that it has a long incubation period in the host and may take several weeks or months to become clinically evident. Thus host may become infectious long before they exhibit clinical signs. Consequently, the mainstay of bTB control in cattle lies in the early detection and removal of Mycobacterium bovis-infected animals. Presently, there is no cost effective treatment available for bTB in cattle. Moreover, eradication of the disease by slaughtering of affected animals is also difficult because of the socio-economic condition of farmers and the social customs or religious taboos (Raghvendra et al., 2010, Verma et al., 2014, Verma et al., 2014a). Therefore an ideal approach to control this zoonotic disease is through genetic selection in livestock populations as genetic gain is cumulative and the genes introduced into a population can persist for many generations. Genetic variation in susceptibility to tuberculosis has been observed in cattle. Prevalence of bTB and severity of its pathology was reported higher in Bos taurus and crossbreds as compared to Bos indicus cattle (Ameni et al., 2007, Phillips et al., 2002). Estimates of the heritability of response to M. bovis PPD (purified protein derivative) was 0.2769, while heritability of TB susceptibility was estimated as 0.18 indicating limited scope for the selection (Bermingham et al., 2009, Brotherstone et al., 2010). Candidate genes with specific roles in immune responses can serve as a useful tool in identifying resistant superior genotypes for the production of resistant animal population (Bermingham et al., 2014, Tsairidou et al., 2014).

TLR9 along with TLR1, TLR2, TLR4 and TLR6 have been recognized as most important pattern recognizing receptors (PRRs) for recognition of pathogen-associated molecular patterns (PAMPs) associated with intracellular pathogens like Mycobacterium bovis and subsequent activation of both innate and adaptive immune response (Zhang et al., 2013, Bafica et al., 2005). TLR9 is located in the endosomal compartment of leukocytes, especially in B cells and monocytes/macrophages (Mortaz et al., 2104) and recognizes the unmethylated Cytosine-phosphate-Guanine (CpG) sequence motifs present in mycobacterial DNA (Hemmi et al., 2000). It acts via MYD88 and TRAF6, leading to NF-kappa-B activation, cytokine secretion and the inflammatory response (Fremond et al., 2004, Scanga et al., 2004). Bovine TLR9 encoding gene is localized on BTA22 and has two exons (Goldammer et al., 2004). Associations between genetic variations and differential expression

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patterns of TLR9 and resistance/susceptibility to TB have been reported in humans and sheep (Velez et al., 2010, Chen et al., 2015; Taylor et al., 2008, Zhong et al., 2009). However, reports on significant association of TLR9 polymorphism with susceptibility/resistance to bTB in cattle are not available. Therefore the objectives of the present study were to genotype a resource population tested for bTB infection and to investigate the potential association between bTB and four SNPs in TLR9 genes in cattle.

MATERIALS AND METHODS

Resource population: Resource population under this investigation comprised of 245 cattle comprising Indigenous (Koshi, Sahiwal, Gir)/Nondescript and crossbred from Shri Mataji Gaushala, Barsana. All animals were kept in same herd and had an equal opportunity of infections. Animals were screened for presence to bTB by Single Intradermal Tuberculin Test (SITT) used to measure Delayed type hypersensitivity (DTH) response. An intradermal inoculation of 0.1 ml of tuberculin PPD antigen on neck region was carried out. The skin thickness was measured with vernier calipers before and 72 hours after inoculation. Based on thickness, cattle were classified into three groups: those showed marked swelling and skin thickness more than 4 mm (Positive), skin thickness < 4 mm and >2 mm (inconclusive) and no reaction > 2 mm (negative). The inconclusive animals were not included in the present investigation. A case and control resource panel of 35 positive and 45 negative animals was developed.

Sample collection and isolation of genomic DNA: From each of case and control animals, 5 ml of blood was collected from jugular vein in tubes containing 2.7% EDTA and stored at -20°C. DNA was isolated from whole blood by using Promega Wizard® Genomic DNA Purification Kit as per recommended protocols. The concentration of DNA was determined using Qubit Fluorometer. Those DNA samples having a minimum concentration of 50 µg/ml were used in further investigation. DNA quality was also assessed by 1% submarine agarose gel electrophoresis. One µl of genomic DNA and Nuclease free water 15.05 -15.175 µl. The cycling program used for amplification having following steps; initial denaturation (94°C for 4 min), followed by 35 cycles of 30 s at 94°C, 30 s at annealing temperature (Table 1), 30 s at 72°C and final extension of 5 min at 72°C. The PCR products were resolved in 2.4% agarose gel and visualized under UV light after staining with ethidium bromide. Restriction digestion was carried out in 25 µl reaction volume which included 20 µL of PCR product, 1.5 U of restriction enzyme, 2.5 µL of 10x buffer and NFW to make volume up to 25 µL and incubated at recommended temperature as proscribed by manufacturer for 16 hours. The restriction enzyme digestion was made at the optimized conditions and the restriction digested products were resolved in 3.5% agarose gel and visualized under UV light after staining with ethidium bromide. Mass genotyping of all case-control resource population for all 7 SNPs was done by using PCR-restriction fragment length polymorphism (PCR-RFLP).

Statistical analysis: Initially in univariate logistic regression analysis, the non-genetic factors like age (two levels), sex (two levels) and breed (two levels) were fitted and found that none of these effects were significantly affecting the Single intradermal tuberculin test result. The association between various allelic variants with bTB tolerance/susceptibility was worked out by suitable statistical techniques using different procedures of SAS 9.3. The PROC LOGISTIC procedure of SAS 9.3 was used to find

<table>
<thead>
<tr>
<th>SNP id</th>
<th>SNP</th>
<th>Position</th>
<th>Primer sequence</th>
<th>Annealing Temperature (°C)</th>
<th>Restriction enzyme</th>
<th>Amplicon size (bp)</th>
<th>Fragment size (bp)</th>
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<td>A/G</td>
<td>+1188</td>
<td>ATCTTCAACGACCTGACCCA AATGCGCAGACTTCCACCT</td>
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<td>BsrI</td>
<td>362</td>
<td>82, 280</td>
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<td>AGACTCAAACCTGCTCTTC GGCATGAAAGCTCTTTGAG</td>
<td>57.5</td>
<td>BseRI</td>
<td>392</td>
<td>251, 141</td>
</tr>
<tr>
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<td>+1433</td>
<td>GTCTGCACGCTGAACTTAC ACGAAGTGCGAGCCATTAAC GGGAGGTGCGACAGCGGTTTGAAG CTGGTCACCGCAGCAAGCATTGGAG</td>
<td>58</td>
<td>BseRI</td>
<td>361</td>
<td>223,138</td>
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<tr>
<td>rs209190268</td>
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<td></td>
<td></td>
<td>250</td>
<td>57, 129, 28, 36</td>
</tr>
</tbody>
</table>
association of allelic and genotypic frequencies with bTB. The Odds Ratio (OR) of genotypes was calculated in affected population versus their contemporary genotypes. The PROC ALLELE procedure of the SAS 9.3 used for the estimation of polymorphism information content (PIC), Hardy Weinberg Equilibrium (HWE) and heterozygosity.

**RESULTS AND DISCUSSION**

Total 245 animals were screened, out of which only 35 cattle (14.28%) were found to be positive for the tuberculin test. All non-genetic factors (breed, age and sex) had non-significant (p < .05) effect on the tuberculin test. The case-control population was genotyped by using PCR-RFLP for the four SNPs in TLR9 gene. Three of SNPs under investigation (rs210982793, rs207807011, rs209190268) revealed polymorphism whereas monomorphism was found in SNP rs55617140. PIC ranged from 0.0767 (rs209190268, rs207807011) to 0.1730 (rs210982793), revealing low to moderate polymorphism at the investigated SNPs. Heterozygosity varied widely from a very low estimate of 0.0833 (rs209190268, rs207807011) to 0.1429 (rs210982793). Similarly, allelic diversity ranged from 0.0799 (rs209190268, rs207807011) to 0.1913 (rs210982793). The chi square test revealed that the population was in HWE for SNPs rs207807011 and rs209190268 while for SNP rs210982793, the population departed from HWE significantly (P<0.05). For the polymorphic SNPs, PIC, Heterozygosity, Allelic diversity and probabilities of being the population in HWE is presented in Table 4. The allelic frequencies and the genotypic frequencies in Case and Control populations at different SNP loci and their effect on susceptibility to infection along with OR have been shown in Table 2 and 3 respectively.

Fig. 1 indicated that at SNP locus rs210982793, three genotypes were identified i.e. CC (392 bp), CT (392 bp, 251 bp, and 141 bp) and TT (251 bp and 141 bp). The probability values showed that the genotype (P = 0.01) as well as allele (P<0.01) had significant effect on occurrence of bovine tuberculosis. Among alleles, OR of C verses T was 0.17 (0.053-0.543; 95% CI). Among genotypes, OR of CC verses TT and CT verses TT were <0.01(<0.01 ->999.99; 95% CI) and <0.01(<0.01 ->999.99; 95% CI) respectively. The odds of CC and CT genotypes verses TT were close to zero, revealing that TT genotypes were relatively more resistant to bTB in comparison to other two genotypes. This SNP lies in the exonic region of TLR9 and is non-synonymous, resulting to change of amino acid Proline to Serine. The amino acid substitution might play a role in susceptibility/ resistance of cattle for bovine tuberculosis. At SNP locus rs207807011, two genotypes i.e. AG (361 bp, 223 bp and 138 bp) and GG (361 bp) were observed (Fig. 2). The probability values showed that the genotype (P <0.01)
as well as allele (P<0.01) had significant effect on occurrence of bovine tuberculosis. The OR of A allele was 0.52(0.11-2.40; 95% CI) revealing lower susceptibility of A allele with bTB in comparison to G allele at TLR9-A1433G locos. The OR of AG allele was 0.19 (0.07 - 0.56; 95% CI) which suggested that AG genotype were less susceptible to tuberculosis as compared to GG. This SNP lies in the exonic region of TLR9 and is non-synonymous, resulting to change of amino acid Glycine to Glutamate. The change of amino acid substitution may have a function in susceptibility/resistance of cattle for bovine tuberculosis. At SNP locus rs209190268, two genotypes i.e. AA (57 bp, 129 bp and 64 bp) and AG (250 bp, 57 bp, 129 bp, 28 bp and 36 bp) were observed. The probability values showed that the genotype (P=0.39) as well as allele (P=0.40) had not significant effect on occurrence of bovine tuberculosis. The OR of A allele G was 0.52(0.11-2.40; 95% CI). The ORs of AA versus AG was 0.51 (0.11 - 2.42; 95% CI)

This is first report of association of SNPs in TLR9 with the susceptibility to bovine tuberculosis. Though TLR9 have specific role in immune response against bTB (Hawn et al., 2007), but association of SNPs from TLR9 gene with susceptibility/resistance had not been reported in cattle. However there are several reports on association of TLR9 polymorphism with tuberculosis in human (Velez et al., 2010, Chen et al., 2015). In cattle, SNP in TLR2 has been reported to be associated with susceptibility to tuberculosis (Bhaladhare et al., 2016). Three out of four SNPs studied for TLR9 gene showed their presence in our Case: Control population and polymorphism at two loci were found to be significantly associated with the susceptibility to bovine tuberculosis. Since, TLR9 have key role in immunity against M. bovis thus more number of SNPs validation in a larger population may reveal biomarkers susceptibility of bovine tuberculosis.

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REFERENCES


