Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2019; 7(3): 248-252

Received: 11-03-2019
Accepted: 13-04-2019

Ashish Bhaladhare
Division of Animal Genetics, ICAR - Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Ramji Yadav
Division of Animal Genetics, ICAR - Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Mohd Baqir
Division of Animal Genetics, ICAR - Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Anuj Chauhan
Division of Animal Genetics, ICAR - Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Arvind Sonwane
Division of Animal Genetics, ICAR - Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Amit Kumar
Division of Animal Genetics, ICAR - Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Ran Vir Singh
Division of Animal Genetics, ICAR - Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Sushil Kumar
Division of Animal Genetics, ICAR - Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Pushpendra Kumar
Division of Animal Genetics, ICAR - Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Subodh Kumar
Division of Animal Genetics, ICAR - Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Bharat Bhushan
Division of Animal Genetics, ICAR - Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Correspondence
Ashish Bhaladhare
Division of Animal Genetics, ICAR - Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Association study of an SNP in TLR1 gene with susceptibility to bovine tuberculosis in cows

Ashish Bhaladhare, Ramji Yadav, Mohd Baqir, Anuj Chauhan, Arvind Sonwane, Amit Kumar, Ran Vir Singh, Sushil Kumar, Pushpendra Kumar, Subodh Kumar and Bharat Bhushan

Abstract
Toll like Receptor 1 (TLR1) gene codes for a host pathogen recognition receptor that plays a vital role in the pathogen recognition and initation of immune response and is a suitable candidate gene for identifying genetic predisposition to infectious diseases like Bovine Tuberculosis (bTB). In the present study, an attempt was made to investigate the genetic polymorphism at SNP locus rs55617193 in TLR1 gene and explore association of genetic variants at this locus with susceptibility to bTB infection in cattle. In a population of bTB affected and healthy cows established using single intradermal tuberculin test; SNP under investigation was found to be polymorphic. SNP locus 55617193 in TLR1 gene was however found to be non- significantly associated with susceptibility to bTB in the study population. These findings need added validation in independent, large case-control population of bTB along with association studies of other SNPs in TLR genes.

Keywords: SNP**,** TLR1, bovine tuberculosis, association, cattle

1. Introduction
Bovine Tuberculosis (bTB) is a chronic, infectious disease of cattle primarily involving the respiratory tract and is caused by *Mycobacterium bovis* (*M. bovis*), which forms part of the *Mycobacterium tuberculosis complex* [1]. *M. bovis* has a wide host range; causes bTB in cattle and a wide range of other domesticated and non-domesticated mammals, including wild animal species such as badgers in the UK and Ireland and possums in New Zealand [2, 3]. bTB infection is prevalent in most of the countries of the world; however it is endemic to Africa and Indian sub-continent [4.5]. Incidence/ prevalence of tuberculosis in cattle at different farms across the country have been reported by several workers [6, 7]. In view of the chronic nature of bTB infection, the foundation of bTB eradication and control in cows lies in the early detection and removal of *M. bovis*-infected animals from the herd. Despite the intensive eradication and control efforts, bTB remains a costly disease for producers and regulatory agencies, with estimates of 50 million cattle being infected worldwide, with an annual economic loss of $3 billion [8]. bTB also presents a barrier to international free trade of livestock apart from consequences in the form of animal health, farmer livelihoods, and natural resources. Additionally, bTB is also a zoonotic disease with associated public health risks in countries where a concerted eradication program is not implemented. In the absence of an acceptable bTB vaccine for use in cattle or wildlife and costly and prolonged treatments; alternative control approaches need to be considered. Genetic studies have suggested that susceptibility to bTB is influenced by the host genetics and that resistance to bTB can be inherited in livestock species. Moderate estimates for heritability for bTB susceptibility trait has been reported in cattle [9-11]. Genetic variation in terms of higher resistance to bTB in *Bos indicus* than *Bos taurus* has been reported [12]. These findings indicate towards a scope for selection and breeding of cows with improved resistance to bTB. Identification of single-nucleotide polymorphism (SNP) in genes involved with the host immune response can be a useful marker in early detection of resistant/susceptible animals. Marker based studies have identified SNPs [13, 14] in genes encoding TLRs [15-20], CARD15 [21], DC-SIGN [22], CD14 [23], TNFα [24], IL12RB1 [25], IFNGR1 [26], SLC11A1 [27], NOS2 [28], CLEC7A [29, 30] and SP110 [31] and QTLs [32]. These genes are either involved in detection of components of mycobacterium and/or subsequent activation of innate and adaptive immune response against

bTB infection. The Bovine Toll Like Receptors (TLRs) genes are host pattern recognition receptors that recognize intrinsic structures of the microorganisms called pathogen-associated molecular patterns (PAMPs) and initiate the development of both innate and adaptive immune responses [33-35]. TLR1-PAMP interactions initiate signalling cascades via transcription factors, such as AP-1 and NF-κB leading to the induction of innate immune responses molecules as pro-inflammatory cytokines, chemokines, and interferon regulatory factors [36]. TLR1 gene, owing to its vital role in the pathogen recognition and immune response, makes it a suitable candidate gene for disease resistance. In a Chinese Holstein population, G1596A polymorphism in the TLR1 gene was found to be associated with bTB infection [13]. However, information on association of TLR1 polymorphism with susceptibility or resistance to bTB in Bos indicus cattle/ crossbreds is much desired. Therefore the present studies were carried out to genotype a case-control population of bTB and ascertain the potential association, if any between susceptibility to bTB and SNPs in TLR1 gene.

2. Materials and Methods

2.1 Development of Case: Control Population

The study population for the present investigation included a total of 245 cows including Indigenous (Koshi, Sahiwal, Gir)/Non-descript and crossbred present at Shri Mataji Gaushala, Barsana in Mathura, Uttar Pradesh. The cows under study were maintained under similar feeding and managemental regime and had equal opportunity of getting infection. Cows were tested for the presence of bTB infection using Single intradermal tuberculin test. In this test, an intradermal injection of 0.1 ml of tuberculin PPD antigen on neck region was done and skin thickness was measured with vernier calipers prior to and 72 h after injection.

The increase in skin thickness after 72 h of intradermal injection of PPD was measured to develop case (tuberculin positive) and Control (tuberculin negative) resource population. On the basis of measured skin thickness, cows were classified into three groups: ones that revealed swelling and skin thickness greater than 4 mm (positive), skin thickness <4 mm and >2 mm (inconclusive), and no reaction <2 mm (negative). The inconclusive cows were not included in the further investigation. A case and control resource panel of 35 positive and 45 negative animals was developed. All the procedures performed in the study involving animals were in accordance with the ethical standards of the Institutional Animal Ethics Committee (IAEC) of ICAR-Indian Veterinary Research Institute (IVRI), Bareilly, India (196/GO/RE/SL/2000/CPCSEA).

2.2 Sample Collection and isolation of Genomic DNA

From panel of 35 positive and 45 negative cows, 5 ml of venous blood was collected in tubes with 2.7% EDTA as anticoagulant. Genomic DNA was extracted from blood samples using Promega Wizard® Genomic DNA Purification Kit as per manufacturer's instructions. The DNA concentration was determined using Qubit Fluorometer. The DNA samples with a minimum concentration of 50 µg/ml were taken for further studies. Quality of the isolated DNA was also checked by ubmarine agarose gel electrophoresis wherein 1 µl of genomic DNA was resolved on 1% agarose gel stained with ethidium bromide and quantification was made by comparing the intensity of the DNA band with the intensity of a known quantity of lambda DNA. Only samples with the thick DNA band and without smearing were selected for further analysis.

2.3 Genotyping of samples for SNP

rs55617193 SNP locus in the TLR1 gene [15] was amplified using Forward: 5'-TAGGCCAAGTATCCAGTGAC-3' and Reverse: 5'-CAGATCCAGGTAGATACAGAG-3'
primers under the optimized PCR condition. The PCR amplification was done in 25 µl volume that comprised of 1 µL of each primers, 1.5 µL $MgCl_2$, 5 µl buffer, 0.2 µL dNTPs, 0.25 µl Taq polymerase, 1 µl genomic DNA and Nuclease free water 15.05 µl. The PCR cycling program consisted of initial denaturation (94 °C for 4 min), followed by 35 cycles of 30 s at 94 °C, 30 s at 57 °C, 30 s at 72 °C and final extension of 5 min at 72 °C. The amplicons were resolved in 3% 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 BslI restriction enzyme, 2.5 µL of 10x buffer and NFW to make volume up to 25 µL and kept for overnight incubation. Restriction fragments were separated in 3% agarose gel and visualized under UV light after ethidium bromide staining. Mass genotyping was performed for the case-control resource population for SNP locus was done by PCR-Restriction Fragment Length Polymorphism (PCR-RFLP).

2.4 Genetic association analysis

Before performing the association anlaysis of genotypes with bTB trait, the non-genetic factors including age (two levels), sex (two levels) and breed (two levels) were fitted initially in univariate logistic regression analysis wherein it was found that none of these effects were significantly affecting the Single intradermal tuberculin test result. At the SNP locus, the genotypes were determined from the restriction fragment patterns of each digested sample in the gels. The gene and genotype frequencies were estimated by the standard procedures and genotype of every animal including homozygote and heterozygote were recorded manually from the autoradiograph. On whole case: control population basis, the number of alleles, their size and frequencies was recorded for the SNP under investigation. The association analysis between various allelic variants with bTB tolerance/susceptibility was carried out in SAS 9.3 program. The PROC LOGISTIC procedure of SAS 9.3 was used to unravel the association of allelic and genotypic frequencies with bTB. The Odds Ratio (OR) of genotypes was calculated in affected population versus their contemporary genotypes. For the estimation of other population genetic parameters like polymorphism information content (PIC), Hardy Weinberg Equilibrium (HWE) and heterozygosity, PROC ALLELE procedure of the SAS 9.3 was employed.

3. Results

In the cattle resource population, 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 SNP locus rs55617193. It showed variability in our Case: Control population. The allelic frequencies and the genotypic frequencies in Case and Control populations at the SNP locus and their effect on susceptibility to infection along with ODDs Ratio (OR) have been shown in Table 1 and 2 respectively. The chi square test revealed that the population

was not in HWE for the SNP locus investigated. SNP locus revealed moderate estimates of PIC and allelic diversity while high estimates were found for heterozygosity. Values of PIC, Heterozygosity, Allelic diversity and probabilities of the population being in HWE for SNP Locus were 0.3733, 0.8929, 0.4965, and <.0001 respectively.

Table 1: Allelic frequency distribution of SNP rs55617193 in TLR1 gene and their association with bTB tested by Single Intradermal tuberculin test

SNP	Allele	Allele frequency		p-Value	Odds ratio (95% CI)
		Case	Control		
rs55617193	A	38 (0.543)	53 (0.541)	0.98	1.01 (0.54 – 1.86)
	G	32 (0.457)	45 (0.459)		1

Table 2: Genotype frequency distribution of SNP rs55617193 in TLR1 gene and their association with bTB tested by Single Intradermal tuberculin test

SNP	Genotype	Genotype frequency		p-Value	Odds ratio (95% CI)
		Case	Control		
rs55617193	A/A	3 (0.086)	5 (0.102)	0.56	>999.99 (<0.01 - >999.99)
	A/G	32 (0.914)	43 (0.877)		>999.99 (<0.01 - >999.99)
	G/G	00	1 (0.021)		1

At SNP locus rs55617193, two alleles i.e. A and G and three genotypes i.e. AA (51 bp, 152 bp), AG (203 bp, 51 bp, 69 bp, 83 bp) and GG (51 bp, 69 bp, 83 bp) were observed in case control population (Fig.1). The A allele was present with a frequency of 0.5428 in case and 0.5408 in control whereas G allele had frequency of 0.457 and 0.459 in case and control population respectively. Similarly the frequency of genotype AA, and AG were 0.086, and 0.914 respectively in case and the frequency of genotype AA, AG and GG were 0.102, 0.877 and 0.020 respectively in control. The probability values showed that the genotype (P=0.56) as well as allele (P=0.98) had non-significant effect on occurrence of bovine tuberculosis. The ODDs ratio of A verse G was 1.01(0.54-1.86; 95% CI), where as ODDs ratio of AA verses GG and AG verse GG were >999.99 (<0.01 - >999.99; 95% CI) and >999.99 (<0.01 - >999.99; 95% CI) respectively (Table 1 and 2).

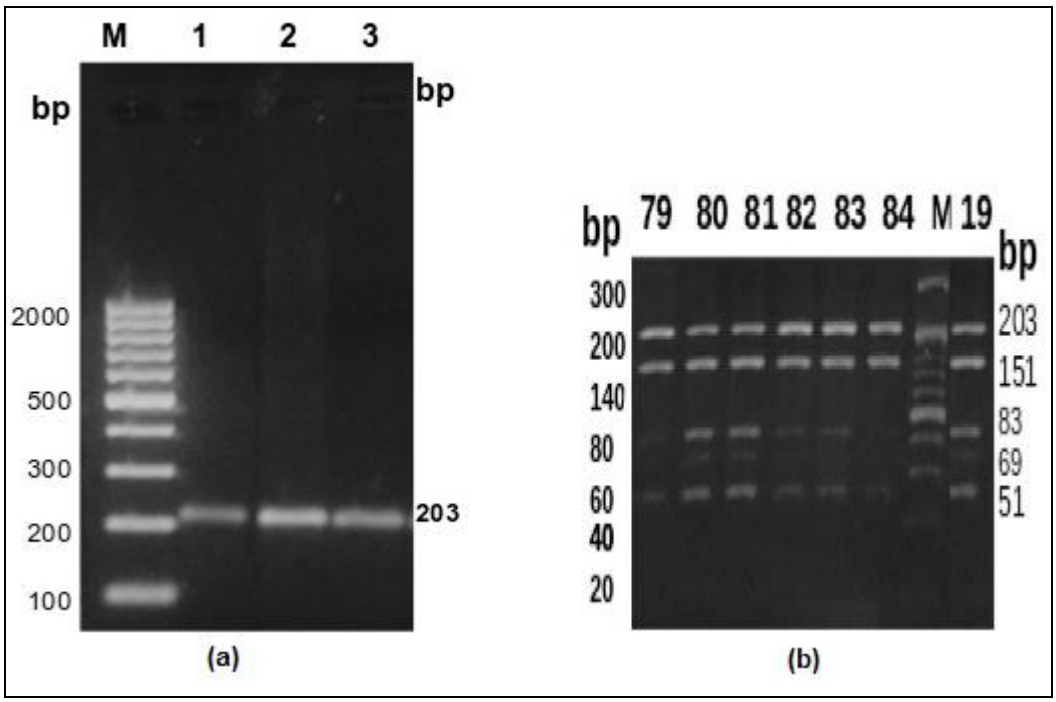

Fig 1: Representative PCR-RFLP profile of rs55617193 locus of TLR1 gene in 3% agarose gel (a) PCR amplicon of 203 bp (b) RFLP patterns (AA, AG and GG) of BslI digested amplicons; Lane M: 100bp ladder

4. Discussion

This investigation was designed to genotype a SNP locus in TLR1 gene and to study its association with susceptibility to bTB in a case-control population of cows established using single intradermal tuberculin test. The SNPs studied for TLR1 gene displayed its presence in our case- control population but the polymorphism at this locus was not found to be significantly ($p>0.05$) associated with the susceptibility to bTB. The SNP locus investigated under the present investigation was located in exonic region of TLR1 genes thus directly governing the encoded proteins with important roles in host immune response to bTB infection. Sun *et al.* [15] investigated this SNP of TLR1 for association with bTB in Chinese Holstein cattle and genetic variability was reported, however the SNP was not significantly associated with bTB susceptibility. A different SNP in TLR1 gene rs55617219 was reported as significantly associated with bTB susceptibility by Sun *et al.* [15] underlying the importance of TLR1 gene in host response to bTB. Further, an SNP at TLR1 (+1380 G/A) was significantly ($P \leq 0.05$) associated with bovine brucellosis in a case control population for bovine brucellosis (Prakash *et al.* [37] established from same resource population as our study. At

TLR1 (+1380 G/A) locus 'A' allele was significantly (P = 0.01) lower than 'G' allele in brucellosis positive animals with its odds ratio of 0.43 (0.22–0.83; 95 % CI). Based on transcriptional profiling of host macrophage mRNA repertoire upon in-vitro *M. bovis* infection, TLRs have been found as among the putative biomarkers for mycobacterial infections in cattle [38-40]. Although SNPs in TLR2, TLR4, TLR9, SP110, CLEC7A along with QTLs have been investigated in Indian cattle breeds and crossbreds for association with mycobacterial infections [15, 17, 18, 28, 29, 30] but no previous report exists on association study of SNPs in TLR1 gene with bTB in Indian cattle breeds and crossbreds. These results also show presence of high genetic diversity in the TLR1 gene among different kinds of cattle. bTB infection has been reported to be present in cattle herds by several workers [41-43] and identification of SNPs associated with susceptibility to bTB could aid in animal selection. Although the studied SNPs in the TLR1 gene was not found to be associated with susceptibility to bTB in our population, other SNPs in TLR1/ signalling pathway genes might influence bTB susceptibility and needs investigation.

5. Conclusions

In this investigation, SNP locus rs55617193 in exonic portion of TLR1 gene was found to be non-significantly associated with the susceptibility to bTB in the resource population of cattle that comprised of bTB affected and healthy cows. However, this study demonstrated the presence of a significant genetic variation in the population for the TLR1 gene, whose encoded protein is vital for generation of an effective immune response against invading *M. bovis*. These findings needs validation in different populations with wider diagnostic panel of tests to identify genetic loci that decide the genetic predisposition to bTB infection in cattle so as to aid in marker based selection for improved cattle health.

6. Acknowledgments

The authors sincerely acknowledge the help and support in the form of funds and important facilities provided by the Director and Joint Director (Research), ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, India to perform this research.

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