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RESEARCH ARTICLE

Single-nucleotide polymorphisms in *CLEC7A*, *CD209* and *TLR4* gene and their association with susceptibility to paratuberculosis in Indian cattle

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Abstract. The aim of this study was to identify the single-nucleotide polymorphisms (SNPs) in bovine candidate genes *CLEC7A*, *CD209* and *TLR4*, and explore the association between these SNPs with the occurrence of bovine paratuberculosis (PTB) disease. For this purpose, 549 animals were screened by a panel of four diagnostic tests, namely Johnin PPD test, ELISA test, faecal microscopy and IS900 blood PCR against *Mycobacterium avium* ssp. *paratuberculosis* (MAP) to develop case–control populations. SNPs were genotyped by polymerase chain reaction-restriction fragment length polymorphism method. Genotypic–phenotypic associations were assessed by the PROC-LOGISTIC procedure of SAS 9.3. Of the seven SNPs; rs110353594 in *CLEC7A* gene and rs8193046 in *TLR4* gene were found to be associated with PTB. For SNP rs110353594, odds of CC and CT genotypes vs TT genotype was 1.543 (0.420–5.667; 95% CI) and 0.284 (0.104–0.774; 95% CI), respectively which means that CT genotype was more resistant than TT and CC genotypes against bovine PTB. For SNP rs8193046, odds of AA and AG genotypes versus GG genotype was 0.947 (0.296–3.034; 95% CI) and 3.947 (1.555–10.022; 95% CI), respectively, i.e. probability for getting an infection in animals with AG genotype was 3.94 times more as compared to GG genotype. Hence, a selection programme favouring CT genotype for rs110353594 and against AG genotype for rs8193046 may be beneficial for conferring resistance against bovine PTB.

Keywords. immune response; paratuberculosis; resistance; single-nucleotide polymorphism; *TLR4* gene.

Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP), the cause of paratuberculosis (PTB) or Johne's disease (JD), is a highly pathogenic *mycobacterium* affecting dairy cattle and other domestic ruminants globally (Singh *et al.* 2013a). PTB is a chronic, infectious, granulomatous, inflammatory bowel disease affecting domestic and wild ruminants leading to persistent diarrhoea, progressive wasting and eventually death (Pieper *et al.* 2015; Gao *et al.* 2018). PTB is considered as the most costliest infectious disease affecting cattle (Singh *et al.* 2013b). It causes heavy economic loss to farmers in the form of increased culling (Benedictus *et al.* 1987; Tiwari *et al.* 2006), decreased milk yield (Benedictus *et al.* 1987), higher death rate and increased susceptibility to other infectious diseases (Tiwari *et al.* 2009). World Organization for Animal Health (OIE) has listed PTB as a notifiable disease of cattle (Correa-Valencia *et al.* 2016). Moreover, PTB is associated with Crohn's disease in human (Correa-Valencia *et al.* 2016), hence it is a matter of zoonotic concern also. Transmission of MAP occurs both by horizontal (through ingestion of milk and feed contaminated with faeces of infected animals) and by vertical means (through *in utero* transmission). Colostrum and milk are an important source of transmission of MAP to newborn calves and human population (Slana *et al.* 2008). Calves become infected soon after the birth due to higher susceptibility to MAP during the first year of life, although clinical symptoms appear at later age (2–6 years) (Prajapati *et al.* 2017). PTB is found to be endemic in India and ∼29.0% (28.6% in buffalo

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and 29.8% in cattle), 31.9% and 23.3% of seroprevalence was reported in northern India, Uttar Pradesh and Punjab, respectively (Singh *et al.* 2000).

At present, there is unavailability of cost-effective treatment for PTB disease and its control is a challenging issue for farmers and veterinarians. Due to the variable sensitivity of different diagnostic tests, culling programmes were not as efficient as expected and proven to be time-consuming and expensive (Nielsen and Toft 2008). Vaccination against MAP, which is cost-effective and considered as the most effective way to control PTB (Fridriksdottir *et al.* 2000; Juste and Perez 2011), did not completely prevent the infection. There are many possible reasons for the low success rates of vaccination against MAP. The main disadvantage of vaccination is that the vaccinated animals cannot be differentiated from the infected animals (Bastida and Juste 2011). In these conditions, new approaches to control livestock diseases are needed. One such approach is the genetic selection for animals that are resistant to the MAP. There is evidence that many genes play a role in resistance against MAP and has a genetic basis that shows considerable additive genetic variation among animals in response to various infectious challenges (Bishop and MacKenzie 2003). Several candidate genes or loci associated with PTB resistance have been identified (Jensen *et al.* 2009; Kumar *et al.* 2011). In support of this strategy, estimates of the heritability for susceptibility to bovine PTB have been found to range from 0.06 to 0.183 (Koets *et al.* 2000; Attalla *et al.* 2010; Küpper *et al.* 2012). Candidate gene studies have primarily focussed on innate immune molecules, important associations between various PTB phenotypes and genetic variants in interferon-gamma, interleukin 10 receptor alpha, nucleotide oligomerization domain 2, peptidoglycan recognition protein 1, solute carrier family 11 member 1, SP110 nuclear body protein, toll-like receptor (*TLR*) 1, *TLR2* and *TLR4* has been identified (Mucha *et al.* 2009; Pinedo *et al.* 2009; Koets *et al.* 2010; Ruiz-Larrañaga *et al.* 2010a; 2010b; Verschoor *et al.* 2010; Pant *et al.* 2011; Sadana *et al.* 2015; Sharma *et al.* 2015; Yadav *et al*. 2014; Kumar *et al.* 2017; 2019a, 2019b).

Dectin-1, a C-type lectin (CTL) receptor encoded by the CTL domain family 7 member A (*CLEC7A*) gene, mapped to *Bos taurus* autosome 5 (Willcocks *et al.* 2006) is an important pattern recognition molecule of the innate immune system that primarily recognizes mycobacterial ligands and fungal cell wall derived β-glucans (Herre *et al.* 2004). It initiates intracellular signalling which results in different cellular responses like the production of pro-inflammatory cytokines and chemokines that participate in cell recruitment and activation (Brown *et al.* 2003; Gantner *et al.* 2003). It also stimulates the production of interleukin-12 (*IL12*) that induces a T-helper 1 cell response and the production of interferon- γ (*IFNG*) (Gantner *et al.* 2003) which is essential to prevent progression of MAP infection (Buza *et al.* 2003; Coussens *et al.* 2004; Weiss *et al.* 2004). The *CD209* also known as dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) molecule is a CTL receptor present on the surface of macrophages and dendritic cells. It works as an adhesion molecule and can initiate innate immunity by modulating *TLR*s. *CD209* shows epistatic interactions with *TLR2* and *TLR4* genes (Ruiz-Larrañaga *et al.* 2017) and found to be associated with the latent PTB (Vazquez *et al.* 2014). *TLR*s are a family of trans-membrane structures capable of recognizing several classes of pathogens including mycobacterium and responsible for the co-ordination of appropriate innate and adaptive immune responses (Quesniaux *et al.* 2004). *TLR*4 has been involved in the recognition of mycobacterial antigens (Quesniaux *et al.* 2004; Yadav and Schorey 2006) mediating cytokine production and stimulation of host defence (Ferwerda *et al.* 2008; Bharati *et al.* 2017). *TLR*4 is associated with increased susceptibility to MAP infection (Mucha *et al.* 2009). Overall, these studies suggest that susceptibility to MAP infection is partially determined by inherent genetic factors and that breeding for increased resistance to PTB may be possible. There is an urgent need to identify a reliable genetic marker that can be applied at field level in the selection of animals resistant to PTB. In keeping view of these facts, the present study was proposed to find the SNPs in *CLEC7A*, *CD209* and *TLR4* genes which are associated with the occurrence of PTB in Indian cattle.

Materials and methods

Experimental site and animals

A total of 549 cattle from two different farms (75% Kamdhenu Gaushala, Nurmahal, Jalandhar, Punjab and 25% from Military Dairy Farm Bareilly, UP) were screened in this study to identify the MAP infected or non-infected animals. Of the 549 animals, 330 were Sahiwal, 26 Tharparkar, 51 Gir, seven Kankrej and 135 Frieswal crossbred. All the crossbred were taken from the military dairy farm and the rest of the cattle were taken from Kamdhenu Gaushala Punjab. Only the adult cattle (above three years of age) were chosen since they had more chance of being exposed to MAP infection than younger animals (Ruiz-Larrañaga *et al.* 2017). All the animal experiments had prior approval of Institutional Animal Ethics Committee (IAEC) of ICAR-Indian Veterinary Research Institute (IVRI), Izatnagar, India, and the experiments were performed in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experimentation in Animals (CPCSEA), Ministry of Environment, Forest and Climate Change, Government of India.

Phenotypic classification of animals based on the health status

The animals were classified considering their health and physical condition visualized in four scales called physical body condition score (PBCS). The coding for PBCS was 4+

for healthy animals with no apparent signs of disease, 3+ for healthy animals with no apparent signs of disease but somewhat weaker than 4+, 2+ for weak and emaciated animals and 1+ for animals with frank clinical signs of disease like diarrhoea and emaciation (Kumar *et al.* 2019a).

Blood collection for serum and DNA isolation

About 5 mL of venous blood were collected from the jugular vein of all the 549 animals under sterile condition into a BD vacutainer coated with EDTA for DNA isolation and 3 mL of blood was collected in BD vacutainer without anticoagulant for serum separation for ELISA test. After the collection of blood, the tubes were kept in the deep freeze at −20°C until the isolation of DNA. Genomic DNA was isolated by phenol–chloroform extraction method as described by Sambrook and Russel (2001). The concentration and purity of DNA were determined by Nanodrop 1000 spectrophotometer. Samples having A260/A280 ratio of 1.7–2.0 were considered as pure DNA and used for further study.

Establishment of case–control population

All experimental animals were screened by a panel of four diagnostic tests, namely Johnin PPD test, ELISA test, faecal microscopy and IS900 blood PCR against MAP to develop case–control population. The Johnin PPD skin test for delayed-type hypersensitivity is a measure of cell-mediated immunity. Johnin PPD prepared from *M. paratuberculosis* containing 1-mg PPD per mL and preserved with 0.5 mL phenol, produced in biological standards division of IVRI was utilized in this study. Intradermal inoculation of 0.1 mL of Johnin PPD, in the shaven site, in the middle of the neck was performed as per manufacturer's protocol. Skin thickness was measured before and after 72 h of inoculation of Johnin PPD injection by vernier callipers. Presence of diffused oedema and increased thickness of about 4 mm and above at the site of injection was considered as positive reactors for PTB.

ELISA was performed for the detection of MAP infection by commercially available PARACHEK ELISA kit using a serum sample of each animal as per manufacturer protocol. The absorbance value of each sample was read using a 450 nm filter and 620 nm as a reference wavelength. The absorbance values were used to calculate the results as,

$$\text{S/P ratio of sample} = \frac{(\text{OD value of sample}) - (\text{OD value of negative control})}{(\text{OD value of positive control}) - (\text{OD value of negative control})}.$$

Results of bovine serum samples which were above or equal to the cut-off of 15% positivity (%P) were considered as positive and below the cut-off of 15% positivity (%P) was considered as negative. Ziehl–Neelsen staining was done to examine the presence of acid-fast bacilli by microscopic examination (Singh *et al.* 2013c). Slides displaying pink colour short rods, indistinguishable to MAP were considered as positive for MAP infection.

IS900 Blood PCR was also done for the detection of MAP infection. About 500 μL of blood collected in 2.7% EDTA was taken in an Eppendorf tube and 1 mL of freshly prepared chilled RBC lysis buffer was added. After mixing well it was centrifuged at 2500 rpm for 10 min. The same step was repeated three or four times until white pellet was obtained. The pellet was dried and 20 μL nuclease-free water was added to it and stored at −20°C until further use. Twenty-five μL PCR reaction mixture was prepared using 12.5 μL GoTaq master mix, 9.5 μL nuclease-free water, 0.5 μL each forward and reverse primers and 2 μL DNA template, as a white pellet. PCR amplification of IS900 elements was done by using specific primers for *M. avium* ssp *paratuberculosis*, forward primer 5′-CCGCTAATTGAGAGATGCGATTGG-3′ and reverse primer 5′-AATCAACTCCAGCAGCGCGGCCTCG-3′ (Vary *et al.* 1990; Ellingson *et al.* 2005). The positive sample showed a product of 229 bp in agarose gel electrophoresis after PCR amplification.

SNP selection and genotyping

In the present study, a total of seven SNPs from *CLEC7A*, *CD209* and *TLR4* genes were selected from NCBI database and suitable primers were designed using online primer designing tool Primer3 to amplify a 300–450 bp fragment containing the targeted SNP. Identification of a restriction enzyme (RE) allowing allele discrimination by *in silico* analysis was done by the programme designated NEBcutter v2.0 software (http://nc2.neb.com/NEBcutter2/) (Vincze *et al.* 2003). The region covered by each primer, SNP database identification number, primer sequence, annealing temperature and amplicon size are depicted in table 1. The genotyping of targeted SNPs was done using PCR-RFLP technique. The RE digested products were checked by electrophoresis on 3.5% metaphore agarose gel stained with ethidium bromide (0.5 μg/mL). Twenty μL of RE digested products were mixed with 4 μL of 6× loading dye and

Table 1. Region covered by each primer, SNP database identification number, primer sequence, annealing temperature and amplicon size for each primer used in the study.

Gene	Primer name	SNP ID[a]	Sequence (5′–3′)	T_a [b] (°C)	Amplicon size (bp)
CLEC7A	CLEC594	rs110353594	F: TCCTGCTTTTGGGTATCTGGT R: TGGTTGCCCAGAGAACAGTC	62	446
CLEC7A	CLEC445	rs41654445	F: ACTGTGTGACATGCTCTTACCT R: AGGCTGATGCCAGTATACGC	61.5	346
CLEC7A	CLEC821	rs110671821	F: ATAGGTGGGCTTGGTTGCTC R: TCTCTCTCCTAAGACAACTGCC	61	325
CD209	CD804	rs208222804	F: TGGCACATGGTCACACAGAA R: AGACACCCTCGACCACTACA	61.2	389
CD209	CD257	rs208814257	F: CTGTAACACATCTGCCATCATTC R: GGGAAGCCCACATTTAACTTTC	56.2	298
TLR4	TLR046	rs8193046	F: TCTTTGCTCGTCCCAGTAGC R: AAGTGAATGAAAAGGAGACCTCA	56.5	384
TLR4	TLR060	rs8193060	F: CAGAAACCTCCGCTACCTTGATA R: TTGACCCACTGCAGGAAACT	59.1	447

[a] SNP database identification number.
[b] Annealing temperature.

Table 2. Restriction enzyme used for each SNP, their incubation temperature and different fragment sizes expected from each amplicon.

SNP ID	Restriction enzyme	Incubation temp. (°C)	Amplicon	1st Homozygous	Heterozygous	2nd Homozygous
rs110353594	*Alw*NI	37	446	446 (CC)	446.305,141 (CT)	305,141 (TT)
rs41654445	*NIa*IV	37	346	346 (TT)	346,262,84 (CT)	262,84 (CC)
rs110671821	*Alu*I	37	325	325 (TT)	325,234,91 (CT)	234,91 (CC)
rs208222804	*Pfl*MI	37	389	389 (AA)	389,248,141 (AG)	248,141 (GG)
rs208814257	*Aci*I	37	298	298 (GG)	298,198,100 (CG)	198,100 (CC)
rs8193046	*Aci*I	37	384	384 (AA)	384,297,87 (AG)	297,87 (GG)
rs8193060	*Alu*I	37	447	447 (TT)	447,318,129 (CT)	318,129 (CC)

carefully loaded in the wells. To analyse the size of the product 100-bp marker was used. Electrophoresis was performed at 2–3 volts/cm for 2 h and then the gel was visualized under a UV transilluminator and documented using gel documentation system. The genotype of each animal was recorded manually from the photograph. Information about the RE, incubation temperature, fragments obtained after RE digestion and genotyping by RE digestion are shown in table 2.

Statistical analysis

The univariate analysis for logistic regression has considered the infection status as categorical response variable (yes/no), and SNPs, breed (2 level), PBCS (2 level) were included as possible explanatory variables. Genotypes were considered as ordinal variables and as class variables with the major homozygous genotype deemed as a baseline. Data were analysed using the PROC LOGISTIC procedure of SAS 9.3 software (SAS Institute, Cary, USA) and odds ratios (OR) with 95% CIs were calculated. The relative risk of incidence among the genotypes was analysed using a univariate logistic regression model:

$$\text{Log}\,[p/(1-p)] = \alpha + \beta_1 H_t + \beta_2 H_0 + \gamma Z.$$

Where α, β_1, β_2 and γ were regression coefficients associated with population, heterozygotes, homozygotes and error parameter, respectively. This model compared heterozygous (H_t) and homozygous (H_0) genotypes and estimated one/two OR, one for H_t and the other for H_0. The PROC ALLELE procedure of SAS 9.3 was used for testing Hardy–Weinberg equilibrium (HWE), linkage disequilibrium (LD), estimation of polymorphism information content (PIC) and heterozygosity of SNPs markers used in present investigation.

Results

Establishment of case–control populations

Of the total samples screened, 50 animals positive for at least two tests selected as case population. Similarly, 50 animals which were negative in all the four specified tests were selected as a control population.

Table 3. Allele frequency distribution and odds ratio of case and control populations for targeted SNPs.

Gene	SNP ID	Allele	Allele frequency		*P* value	Odds ratio (95% CI)
			Case *N* (%)	Control *N* (%)		
CLEC7A	rs110353594	C	23 (23)	27 (27)	0.5140	0.808 (0.425–1.534)
		T	77 (77)	73 (73)		1.00
	rs41654445	C	86 (86)	92 (92)	0.1802	0.534 (0.214–1.336)
		T	14 (14)	8 (8)		1.00
	rs110671821	C	5 (5)	0 (0)	0.979	>999.999 (<0.001–>999.999)
		T	95 (95)	100 (100)		1.00
CD209	rs208222804	A	24 (24)	36 (36)	0.0655	0.561 (0.304–1.038)
		G	76 (76)	64 (64)		1.00
	rs208814257	C	73 (73)	69 (69)	0.5338	1.214 (0.659–2.239)
		G	27 (27)	31 (31)		1.00
TLR4	rs8193046	A	37 (37)	30 (30)	0.295	1.370 (0.760–2.471)
		G	63 (63)	70 (70)		1.00
	rs8193060	C	27 (27)	28 (28)	0.8742	0.951 (0.511–1.769)
		T	73 (73)	72 (72)		1.00

N, number of particular allele (C/T/A/G); %, percentage in case and control population.

Table 4. SNP ID, genotype, genotype frequency distribution in case and control population, *P* value and OR with 95% CI of targeted SNPs.

Gene	SNP ID	Genotype	Genotype frequency		*P* value	Odds ratio (95% CI)
			Case *N* (%)	Control *N* (%)		
CLEC7A	rs110353594	CC	8 (16)	4 (8)	0.0249*	1.543 (0.420–5.667)
		CT	7 (14)	19 (38)		0.284 (0.104–0.774)
		TT	35 (70)	27 (54)		1.00
	rs41654445	CC	39 (78)	42 (84)	0.9907	<0.001 (<0.001–>999.999)
		CT	8 (16)	8 (16)		<0.001 (<0.001–>999.999)
		TT	3 (60)	0 (0)		1.00
	rs110671821	CT	5 (10)	0 (0)	0.9678	>999.999 (<0.001–>999.999)
		TT	45 (90)	50 (10)		1.00
CD209	rs208222804	AA	6 (12)	12 (24)	0.2811	0.406 (0.134–1.231)
		AG	12 (24)	12 (24)		0.813 (0.313–2.107)
		GG	32 (64)	26 (52)		1.00
	rs208814257	CC	33 (66)	27 (54)	0.1665	0.978 (0.339–2.821)
		CG	7 (14)	15 (30)		0.373 (0.103–1.359)
		GG	10 (20)	8 (16)		1.00
TLR4	rs8193046	AA	6 (12)	10 (20)	0.0092**	0.947 (0.296–3.034)
		AG	25 (50)	10 (20)		3.947 (1.555–10.022)
		GG	19 (38)	30 (60)		1.00
	rs8193060	CC	1 (2)	4 (8)	0.3388	0.271 (0.028–2.597)
		CT	25 (50)	20 (40)		1.354 (0.603–3.039)
		TT	24 (48)	26 (52)		1.00

N, number of animal having particular genotype in case and control population.
*Significant at 5%.
**Significant at 1%.

Effect of SNP

In the present investigation, all the seven SNPs showed polymorphic pattern on genotyping by PCR-RFLP. Except SNP rs110671821 of *CLEC7A* gene which revealed two genotypes, all the other six SNPs showed three possible genotypes. The allele and genotype frequencies of all the SNPs under investigation were estimated and their OR have been depicted in tables 3 and 4, respectively.

Effect of SNPs in CLEC7A gene

Of the three SNPs in *CLEC7A* gene, only rs110353594 was found to be significantly associated with the occurrence of

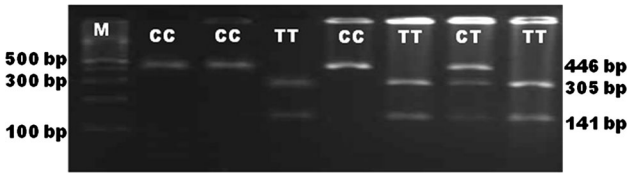

Figure 1. Genotypic profile of SNP rs110353594 in *CLEC7A* gene by PCR-RFLP with 100-bp marker (M represents marker lane).

PTB in our population. The frequencies of common allele in rs110353594, rs41654445 and rs110671821 were 0.77, 0.86 and 0.95, respectively in case and 0.73, 0.92 and 1.0, respectively in control population. The SNP rs110353594 yielded three genotypes, namely CC, CT and TT with frequencies 0.16, 0.14 and 0.70, respectively in case population and 0.08, 0.38 and 0.54, respectively in control population. The logistic regression analysis revealed that these genotypes differ significantly ($P < 0.05$) in case and control populations. The genotypic profile of SNPs rs110353594 is depicted in figure 1. CT genotype was more abundant in the control population (38%) as compared with the case (14%).

Effect of SNPs in the CD209 gene

Two SNPs in *CD209* gene, namely rs208222804 and rs208814257 were included in this study to find the association with bovine PTB. Both the SNPs were found to be polymorphic in case and control populations but failed to show any significant association with the occurrence of PTB in cattle. The SNP rs208222804 yielded three genotypes, namely AA, AG and GG with frequencies 0.12, 0.24 and 0.64, respectively in case population and 0.24, 0.24 and 0.52, respectively in control population.

Effect of SNPs in TLR4 gene

Two SNPs in *TLR4* gene, namely rs8193046 and rs8193060 genotyped by PCR-RFLP method was checked for association with PTB. Both the SNPs were found to be polymorphic in case and control populations and yielded three genotypes. The SNP rs8193046 yielded three genotypes, namely AA, AG and GG with genotype frequencies 0.12, 0.50 and 0.38, respectively in case population and 0.20, 0.20 and 0.60, in control population. These genotypes were significantly ($P < 0.01$) different in case and control population. The genotypic profile of SNP rs8193046 is depicted in figure 2. SNP rs8193060 yielded three genotypes, namely CC, CT and TT with frequencies 0.02, 0.50 and 0.48, respectively in case population and 0.08, 0.40 and 0.52 in control population. There is no significant difference ($P > 0.05$) in these genotypes between case and control populations.

HWE and LD test

The PIC, heterozygosity, allelic diversity and HWE have been estimated at all polymorphic SNPs loci and are shown in table 5. The PIC ranged from 1.96% for rs8193046 in *TLR4* to 34.63% for rs208814257 in the *CD209* gene revealing a low to moderate polymorphism at these loci in a case–control population of the present study. Accordingly, at the same locus, heterozygosity ranged from 2% to 35% in case–control population in the present study. In this study, of the seven SNPs, three namely rs110671821, rs208222804 in *CLEC7A* and rs208814257 in *CD209* gene had shown significant deviation from HWE and the rest three were in HWE. This deviation from HWE may be due to the mandate of *gaushala* of keeping migratory animals (Prakash *et al.* 2014). The LD between different SNPs were tested by chi-square(χ^2) probabilities, which revealed that some SNPs were significantly associated with other non-significant SNPs but the loci which were significantly associated with occurrence of PTB had not shown significant LD with each other hence the chances of getting haplotypes from SNPs significantly associated with PTB were rare.

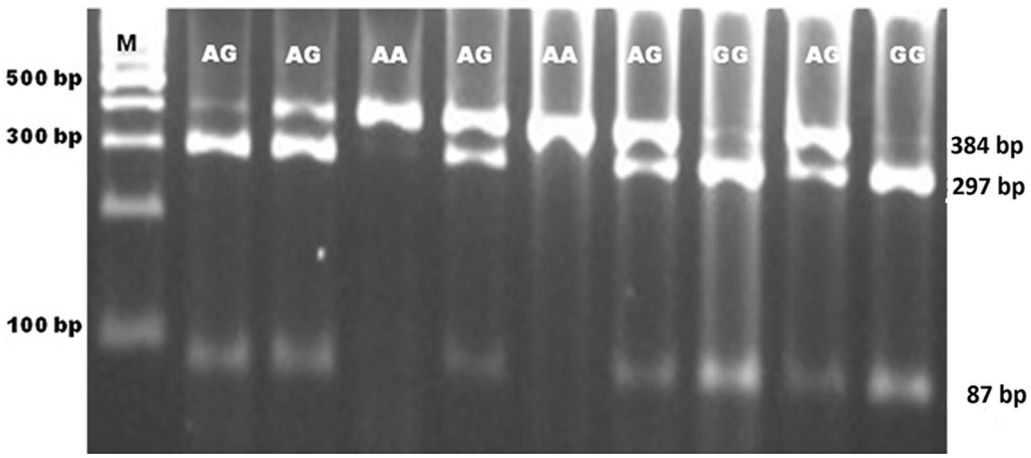

Figure 2. Genotypic profile of SNP rs8193046 in *TLR4* gene by PCR-RFLP with 100-bp marker (M represents marker lane).

Table 5. SNP ID, number of animals (*N*) taken for the study, alleles, PIC, heterozygosity, allelic diversity and Hardy–Weinberg equilibrium (HWE) χ^2-probability as estimated by chi square method for all the loci under study.

SNP ID	*N*	Number of alleles	PIC	Heterozygosity	Allelic diversity	HWE (χ^2-probability)
rs110353594	100	2	0.1766	0.16	0.1958	0.0966
rs41654445	100	2	0.0476	0.05	0.0487	1
rs110671821	100	2	0.3318	0.24	0.42	<.0001**
rs208222804	100	2	0.327	0.22	0.4118	<.0001**
rs208814257	100	2	0.3463	0.35	0.4456	0.044*
rs8193046	100	2	0.0196	0.02	0.0198	1
rs8193060	100	2	0.2561	0.31	0.3016	1

*Significant at 5%.
**Significant at 1%.

Discussion

The present study was undertaken with the objectives to investigate an association of SNPs in candidate genes with the occurrence of bovine PTB so that reliable genetic marker can be identified to screen susceptible animals from the population. The alleles of all seven SNPs investigated in our population were not differing significantly between case and control population, however genotypic frequencies in SNPs rs110353594 in *CLEC7A* gene and rs8193046 in the *TLR4* gene were significantly different between PTB affected and control populations. The SNP rs110353594 in *CLEC7A* gene showed CC, CT and TT genotypes which were significantly different in case and control populations where the odds of CC and CT genotypes vs TT genotype was 1.543 (0.420–5.667; 95% CI) and 0.284 (0.104–0.774; 95% CI) respectively, i.e. probability of getting infection in individuals with CT genotype is three times lesser than the animal with TT genotype and five times lesser than animal with CC genotype. The OR also indicates that CT genotype was more resistant than TT and CC genotypes against bovine PTB. The OR of C allele compared with T allele was 0.80 (0.425–1.534; 95% CI), i.e. the probability for getting an infection in animals with C allele is comparatively lesser in animals with T allele.

Similar to our findings in PTB affected animals, SNPs rs110353594 and rs41654445 in *CLEC7A* genes were significantly associated with the occurrence of PTB in Indian cattle (Kumar *et al.* 2019a). The genotypes in these SNPs were significantly different in case and control population. Pant *et al.* (2014) have also identified a significant association between SNP c.589A/G ($P = 0.008$) of *CLEC7A* gene and MAP infection status in Canadian Holstein cattle. *CLEC7A* gene plays a very important role against mycobacterial infection and provides immunological defence against MAP. It is also involved in interleukin-1β synthesis and maturation which is an important pro-inflammatory cytokine in immunity. There are several reports of polymorphism in *CLEC7A* gene in different species. Yak *CLEC7A* gene has shown a polymorphic pattern in exons 4 and 5 and a total of three SNPs had been reported (Yang *et al.* 2011). In ovine *CLEC7A* gene, polymorphism was investigated and a total of nine SNPs were found in exons 4–6 and affects the immune response of ovine (Zhou *et al.* 2010). In pig, the polymorphism in the coding region of *CLEC7A* gene had been detected and 10 SNPs were found, of which four were synonymous and six were nonsynonymous (Shinkai *et al.* 2016).

SNPs in the *CD209* gene failed to show a significant difference ($P > 0.05$) in the allele and genotypes of the case–control populations. SNP rs208222804 showed AA, AG and GG genotypes where the odds of AA and AG genotypes vs GG genotype was 0.406 (0.134–1.231; 95% CI) and 0.813 (0.313–2.107; 95% CI), respectively. Similarly odds of disease occurrence in animals having A (OR 0.56) allele were almost two times lower as compared to G allele. Thus, the animals having A allele and AA genotype were at lower risk for PTB infection, but this SNP did not exhibit a significant association with PTB. SNP rs208814257 yielded three genotypes namely, CC, CG and GG with frequencies 0.66, 0.14 and 0.20, respectively in case population and 0.54, 0.30 and 0.16, respectively in control population. There is no significant difference ($P > 0.05$) in these genotypes between case and control populations. The odds of CC and CG genotypes vs GG genotype was 0.978 (0.339–2.821; 95% CI) and 0.373 (0.103–1.359; 95% CI), respectively, i.e. the probability of getting an infection in individuals with CG genotype is three times lesser than the animal with GG genotype.

The failure to show a significant association of *CD209* gene with bovine PTB in our population was in agreement with the Vázquez *et al.* (2014) who reported that none of the analysed SNPs in the *CD209* genes were found to be associated with patent PTB. They also failed to show an association of SNP rs208814257 with the occurrence of PTB. However, SNP rs208222804 was found to be associated with latent PTB (OR 0.64, 95% CI = 0.48–0.86). Chang *et al.* (2012) found an association of two SNPs in the promoter region of *CD209* with human tuberculosis and hence protective nature of *CD209* gene against mycobacterial infection. Five SNPs in *CD209* gene had shown epistatic interaction with *TLR2* and *TLR4* gene among PTB positive cattle which support the role

of *CD209* gene in the innate and adaptive immune response against MAP (Ruiz-Larrañaga *et al.* 2012, 2017). Although in the present study, *CD209* gene failed to show a definite association with PTB infection in Indian cattle, the analyses indicated a probable connection between SNPs in the *CD209* gene and PTB. Hence, further investigation in a larger population and with new SNPs is needed to show the association of *CD209* gene with PTB in cattle.

The SNP rs8193046 in *TLR4* gene showed AA, AG and GG genotypes which were significantly different in case and control populations, where the odds of AA and AG genotypes vs GG genotype was 0.947 (0.296–3.034; 95% CI) and 3.947 (1.555–10.022; 95% CI), respectively, i.e. probability for getting infection in animals with AG genotype was 3.94 times more as compared to GG genotype. This hypothesis was supported by the observation of gene frequency and OR between case and control. Gene frequencies of A and G in the case and control populations were 0.37, 0.63 and 0.30, 0.70, respectively. The odds of 'A' allele vs 'G' allele was 1.370 (0.760–2.471; 95% CI). Here, one more interesting finding need to be concerned is that animals with AG genotype, i.e. heterozygote were more abundant in the case than control. It is a possible example of a heterozygous disadvantage as the odds of AG were greater than both homozygotes.

Our findings are in a similar line with Kumar *et al.* (2019b), they also reported the significant association of SNP rs8193046 in *TLR4* gene with the occurrence of PTB. A significant association with MAP infection was reported for two missense mutations in *TLR4* and four specific nucleotide substitutions were detected in individuals showing clinical signs (Mucha *et al.* 2009). Cinar *et al.* (2018) have also investigated polymorphism in *TLR1*, *TLR4* and *TLR9* for their association with susceptibility to PTB in Turkish Holstein and crossbred animals and found that *TLR1* (+1380 G/A) mutation showed an association with MAP infection but failed to show the association of *TLR4* with PTB. The possible reason for this may be that the investigated markers were not in linkage disequilibrium with the genes affecting disease susceptibility and also the SNP failed to show any significant difference between case and control populations. *TLR4* gene has an important role in innate immunity and recognition of MAP. Sharma *et al.* (2015) had explored the association of SNPs in *TLR4* gene with PTB in Canadian Holstein cows, and found that the haplotype of two SNPs was significantly associated with susceptibility to PTB and hence supported the *TLR4* involvement in susceptibility to PTB. Three SNPs in *TLR4* gene, which were tightly linked to each other, were found to be associated with susceptibility to PTB in Spanish Holstein cattle (Ruiz-Larrañaga *et al.* 2011). One of these three SNPs c.-226G > C can alter the expression of *TLR4* hence contribute to the variable response of the individual animal to the MAP infection.

The effect of non-genetic factors like PBCS, breed and age was investigated for its association with the occurrence of PTB and it was found that none of these factors was significantly ($P \leq 0.05$) associated with the occurrence of PTB. All the animals in the present study have an equal opportunity of getting MAP infection because they were kept in a loose housing system during the day and housed together at night. Our results are in accordance with the several earlier reports. Sadana *et al.* (2015) and Kumar *et al.* (2019a) did not find a significant effect ($P > 0.05$) of breed and age on the occurrence of PTB. Thus in our population, all breeds were at equal risk of getting MAP infection. Sadana *et al.* (2015) also found a similar result where the odds for animals with poor physical conditions (0.92) were also not much different from healthy animals. However, PBCS had significant ($P < 0.01$) difference among poor PBCS animals from healthy animals and animals with poor physical conditions were at higher risk of getting MAP infection (Kumar *et al.* 2017). Despite the non-significant effect of age, lower age group was at a higher risk of infection than higher age group animals. This finding is in accordance with an earlier study of Chase *et al.* (2008), where they showed that young calves are most susceptible, most likely due to their lower immune competence. Kumar (2015) also reported that lower age group was at a higher risk of infection than higher age group animals.

To enhance the efficiency of PTB control programme, new strategies are needed which should be cost-effective and less time-consuming. One such strategy may be the SNPs genotyping which can precisely evaluate the immune-pathological interaction of host and pathogen for control purposes. Since the sero-prevalence rate of PTB in cattle is 23.3% in Punjab (Singh *et al.* 2000) from where our resource population belongs, we could obtain less number of PTB infected animals. Further, we have used a panel of diagnostic test to establish our case–control population and to avoid inclusion of any false positive and false negative, animals positive in two or more test were included in the case and animals negative in all test were included in the control population. This restricted our resource population to 100 animals and being the limitation to this study. However, study in a larger resource population will warrant the strength of association reported in this study.

In conclusion, the results of the present study suggest that *CLEC7A* and *TLR4* genes play a pivotal role in determining the risk to MAP infection in Indian cattle. SNP rs8193046 in *TLR4* gene produces similar results with the previous work in a different resource population, hence it reaffirms this SNP can serve as a reliable marker for identifying MAP susceptible animals and AG genotype of this SNP can be utilized in future breeding programmes to eliminate MAP susceptible animals. Other significant SNPs rs110353594 in *CLEC7A* gene found in our study population can also be included in the marker panel after further validation in a larger independent population.

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