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Bovine IL12RB1, IL12RB2, and IL23R Polymorphisms and Bovine Tuberculosis (bTB) Infection Status

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Mycobacterium tuberculosis **complex (MTC) causes bovine tuberculosis (bTB), a chronic infectious disease of Bovidea family characterised by the formation of granulomas in various organs. The host cytokine machinery plays an important role in providing protection against bTB infection. Therefore, the objective of the present study was to assess whether polymorphisms in candidate genes encoding important cytokine receptors are associated with bTB infection status of dairy cattle. Seven reported polymorphisms in interleukin (IL) genes were genotyped by using PCR-RFLP in a resource population of 84 dairy cattle with known bTB infection status and genotype analysis were performed individually for each gene via PROC LOGISTIC in the statistical software SAS. The three single nucleotide polymorphisms (SNPs) in IL12RB1 (rs210615796) and IL12RB2 (rs208115312 and rs135336138) were found to be monomorphic while for rest of the SNPs polymorphism in IL12RB1 (rs132856935 and rs110233569), IL12RB2 (rs211644228) and IL23R (rs42497589) genes was observed. This suggest the presence of these SNPs in the resource population. The population was in Hardy-Weinberg equilibrium (HWE) for all the SNPs studied. A moderate heterozygosity, polymorphism information content (PIC) value and allelic diversity were estimated at rs110233569 while corresponding low values for other SNPs observed. However, these SNPs were found to be non-associated with the bTB infection status of the resource population. These results need further validation in independent, large resource population and biological characterization is warranted with more effective case-control identification. In addition, other SNPs in these concerned genes could be further exploited for association studies.**

Key words: Bovine tuberculosis, SNPs, Interleukin genes, PCR-RFLP, heterozygosity, PIC value and allelic diversity.

Tuberculosis (TB) is a complex and multi-species disease which can be of three types, bovine, avian and human TB[1,2]. *M. bovis* is a member of *Mycobacterium tuberculosis* complex (MTC) and based upon 16S ribosomal RNA sequence studies it shared more than 99.95% identity with other members of MTB complex[1,3,4,5]. Bovine tuberculosis (bTB) is a chronic infectious disease of cattle, buffaloes and many wild species. It is characterised by progressive development of tubercles or nodular granuloma with resultant caseations and calcification in many of the vital organs especially in the lungs, lymph nodes, intestine and kidney except skeletal muscles[5,6,7]. Bovine tuberculosis

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(bTB) is caused by *Mycobacterium tuberculosis* complex comprising of *M. bovis, M. caprae* (mostly infect goats) and *M. pinnipedii* (usually infect fur seals and sea lions), which is an aerobic, gram-positive, non-motile, non-sporulating, acid-fast, rod shaped slow-growing organism, obligate intracellular parasite[1,3,8]. However, *M. bovis* is the most universal pathogen among mycobacteria and affects many vertebrate animals of all age groups[5,6]. Bovine tuberculosis (bTB) is the zoonotic disease transmitted from animal to human and makes a great economic loss due to high cost of eradication programs and has serious consequences for movements of animals, constrains in international trade of animals and their products, biodiversity, public health and significant economic effect[5,9,10,11]. In developed countries, bTB in animals is a rarity with occasional severe occurrences in small groups of herds. However, in developing countries such as in Africa 46% incidence, 44% of Asian and 35% of the South American and the Caribbean countries, sporadic occurrences and enzootic occurrences of bTB have been reported[12].

Bovine TB is a major public health problem in India, however little data available regarding transmission of bTB and its impact on human health. Adding to the problem there is no cost effective treatment for bTB, so generally treatment is not recommended. 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[7,10,13,14]. Therefore an ideal approach to control this zoonotic disease is through development of genetic resistance. Candidate gene approach can serve as a useful tool in identifying resistant superior genotypes for the production of new resistant animal population[15,16,17]. The host cytokine response is an important target in this regard, as it plays a major role in regulating the defence against *Mycobacterium spp.*[18].

Since bTB is an obligate intracellular pathogen that multiplies within the macrophages, pro-inflammatory and cytotoxic responses driven by Th1 cytokines are necessary to control the spread of infection. IL-12 is an important pro-inflammatory cytokines in this regard as they can steer the host immune response towards a Th1 bias favouring macrophage effector function[19,20,21]. IL-23 promotes the pro-inflammatory response and plays role in the development and differentiation of Th17 cells[22,23]. Genetic variants in cytokine and its receptors such as IL23R, IL12RB1 and IL12RB2 have been associated with Johne's disease (JD)[24] that in many respect parallels TB in cattle. Therefore, the objective of this study was to genotype a resource population tested for bTB infection and to evaluate association with bTB infection status for previously reported single nucleotide polymorphisms (SNPs) in the gene encoding IL23R, IL12RB1 and IL12RB2.

MATERIALS AND METHODS

Resource population

More than 245 animals from Shri Mataji Gaushala, Barsana; Cattle Farm from Atta, Jalon and Akha village, Bareilly, U.P., India were screened for presence to bovine tuberculosis. Single Intradermal Comparative Cervical Tuberculin (SICCT) test was done and increase in thickness of skin after 72 h of intradermal injection of tuberculin antigen noted to develop in case and control resource panel as per standard protocol. Then 35 positive and 49 negative animals were selected as case-control panel and 6 ml of blood was collected from jugular vein in tubes containing EDTA were stored at -20°C. Promega wizard kit was used to isolate genomic DNA. The concentration was determined using Qubit Flurometer. Concentration of sample = QF value X (200/Y)

Where,

QF value = Value given by the Qubit fluorometer

Y = Number of microliters of sample added to the assay tube.

One microliter of genomic DNA was resolved on 1% agarose gel stained with ethidium bromide or SYBR® Safe DNA gel stain and quantification was made by comparing the intensity of the band with the intensity of a known quantity of lambda DNA. Only thick DNA band and without smearing were chosen for further processing.

SNP genotyping

Primers for the 7 reported SNPs in IL12RB1 (rs132856935, rs110233569 and rs210615796), IL12RB2 (rs208115312, rs135336138 and rs211644228) and IL23R (rs42497589) genes were

designed using Oligoanalyser for amplification of the loci. The detail of primers and restriction enzymes are being presented in Table 1. Concerned amplicons were amplified under optimized polymerase chain reaction (PCR) conditions. The PCR product are resolved in 2.4% agarose gel and visualized under UV light after staining with ethidium bromide. The restriction enzyme digestion was made at the optimized conditions and the restriction digested products were resolved in 4% to 5% agarose gel and visulaized under UV light after staining with ethidium bromide. Mass genotyping of all case-control resource population was done by using PCR-restriction fragment length polymorphism (PCR-RFLP).

Statistical analysis

The PROC ALLELE procedure of the SAS 9.3 software was used for the estimation of polymorphism information content (PIC), Hardy-Weinberg equilibrium (HWE) and heterozygosity. The PROC LOGISTIC procedure of SAS 9.3 software was also used to find association of allelic and genotypic frequencies with bovine TB.

RESULTS

PCR-RFLP assay

The case-control population was genotyped by using PCR-RFLP for the seven SNPs in IL receptor genes. The three SNPs in IL12RB1 (rs210615796) (Fig. 1A) and IL12RB2 (rs208115312 and rs135336138) (Fig. 1B) were found to be monomorphic while for rest of the SNPs polymorphism was found. This suggest that the presence of these SNPs in our resource population. The population was in HW equilibrium (HWE) for all the SNPs studied. A moderate heterozygosity, PIC value and allelic diversity were estimated at rs110233569 while corresponding low values for other SNPs observed (Table 2). The allelic and genotypic frequencies of the SNPs used for analysis are presented in Table 3 and 4.

At rs132856935 the frequency of Tallele was 0.9714 in case and 0.9592 in control, C allele had the frequency of 0.0286 and 0.0408 in case and control population respectively. Similarly the frequency of genotype TT, CT and CC were 0.9429, 0.0571 and 0 in case and 0.9184, 0.0816 and 0 in control, respectively (Fig. 3A). At rs110233569 the frequency of G allele was 0.6857 in case and 0.7653

Table 1. Details of single nucleotide polymorphisms (SNPs), primers and restriction enzymes (RE)

SNPs I.D	Chromo some no.	SNP	Primer Sequence (5'-3')	AT (°C)	RE	Fragments	Change of amino acid
rs132856935 (IL12RB1)	7	C/T	GGAGCCCTTCCTATTGATCC TCTGCTTCCTGTCCTTCAAG	57	HpyCH 4III	324,200, 124	Met- Thr
rs110233569 (IL12RB1)	7	C/G	TAGAGTTTGCCGAGAGAGTG TGGTAATGGAGGATGCAGG	57	AluI	312, 201, 111	Gln-His
rs210615796 (IL12RB1)	7	A/G	GGTTAGAGTTTGCCGAGAGAG CAGACTTGTGCGAGTAGGAG	57	SmaI	444, 275, 169	Arg-Glu
rs208115312 (il12rb2)	3	C/T	ATCTGGGCGGTGCTACTAAG AGCTGTGGAGCCTGAATCAC	59	NlaIII	249,150, 99	Val-Met
rs135336138 (il12rb2)	3	G/T	TCCTGCCTGAAGTTTCCTTG TTTGCTGGGTTGGGTAGTTG	58	HaeIII	353,205, 148	Thr-Pro
rs211644228 (il12rb2)	3	A/G	TGGAACCCTGAACAAGTTGG CTTGTCCTGTCTCTGCTGTG	58	PstI	317, 199, 118	Arg-Cys
rs42497589 (il23r)	3	C/T	CCCTCTTCAGATTGCAAGGC ACTTGGGAAACAATGCCAGG	59	HpaII	380,246, 134	Pro-pro

in control, C allele had the frequency of 0.3143 and 0.2347 in cases and control population respectively. Similarly the frequency of genotype GG, CG and CC were 0.4571, 0.4571 and 0.0857 in case and 0.5918, 0.3469 and 0.0612 in control, respectively (Fig. 3B).

At rs211644228 the frequency of G allele was 0.9857 in case and 0.9898 in control, A allele had the frequency of 0.0143 and 0.0102 in case and control population respectively. Similarly the frequency of genotype GG, AG and AA were 0.9714, 0.0286 and 0 in case and 0.9796, 0.0204 and 0 in control, respectively (Fig. 4A). At rs42497589 the frequency of T allele was 0.0286 in case and 0.0204 in control, C allele had the frequency of 0.9714 and 0.9796 in case and control population respectively. Similarly the frequency of genotype TT, CT and CC were 0, 0.0571 and 0.9429 in case and 0.0204, 0 and 0.9796 in control, respectively (Fig. 4B).

Table 2. Polymorphism at different SNPs and Chi-square probabilities for HWE

Locus	Alleles	Genotypes	PIC	Hetrozygocity	Allelic Diversity	P value forHWE
rs132856935 (IL12RB1)	2	84	0.0665	0.0714	0.0689	0.7343
rs110233569(IL12RB1)	2	84	0.3153	0.3929	0.3922	0.9881
rs211644228(IL12RB2)	2	84	0.0232	0.0238	0.0235	0.9121
rs42497589(IL23R)	2	84	0.0454	0.0238	0.0465	0.0001

Table 3. Allelic frequencies and their association with susceptibility to bTB in case-control population

SNP	Allele	Allele frequency		P value	Odds ratio
		Case	Control		(95% CI)
rs42497589(IL23R)	T	2(2.86)	2(2.04	0.73	1.00
	C	68(97.14)	96(97.96)		0.70 (0.09 to 5.15)
rs132856935(IL12RB1)	T	68(97.14)	94(95.92)	0.66	1.00
	C	2(2.86)	4(4.08)		0.69 (0.12 to 3.88)
rs110233569(IL12RB1)	G	48(68.57)	75 (76.53)	0.25	1.00
	C	22(31.43)	23 (23.47)		1.49 (0.75 to 2.97)
rs211644228(IL12RB2)	G	69(98.57)	97(98.98)	0.81	1.00
	A	1(1.43)	1(1.02)		1.40 (0.08 to 22.86)

Table 4. Genotypic frequencies and their association with susceptibility to bTB in case-control population

SNP	Genotype	Genotype frequency		P value	Odds ratio
		Case	Control		(95% CI)
rs42497589(IL23R)	TT		1 (2.04)	0.09	1.00
	CC	33 (94.29)	48 (97.96)		>999.999 (<0.001 - >999.999)
	CT	2 (5.71)			>999.999 (<0.001- >999.999)
rs132856935(IL12RB1)	TT	33 (94.29)	45 (91.84)	0.66	1.00
	CT	2 (5.71)	4 (8.16)		0.68 (0.11 to 3.94)
rs110233569(IL12RB1)	GG	16 (45.71)	29 (59.18)	0.47	1.00
	CC	3 (8.57)	3 (6.12)		1.81 (0.32 to 10.04)
	CG	16 (45.71)	17 (34.69)		1.70 (0.68 to 4.26)
rs211644228(IL12RB2)	GG	34 (97.14)	48 (97.96)	0.81	1.00
	AG	1 (2.86)	1 (2.04)		1.41 (0.08 to 23.36)

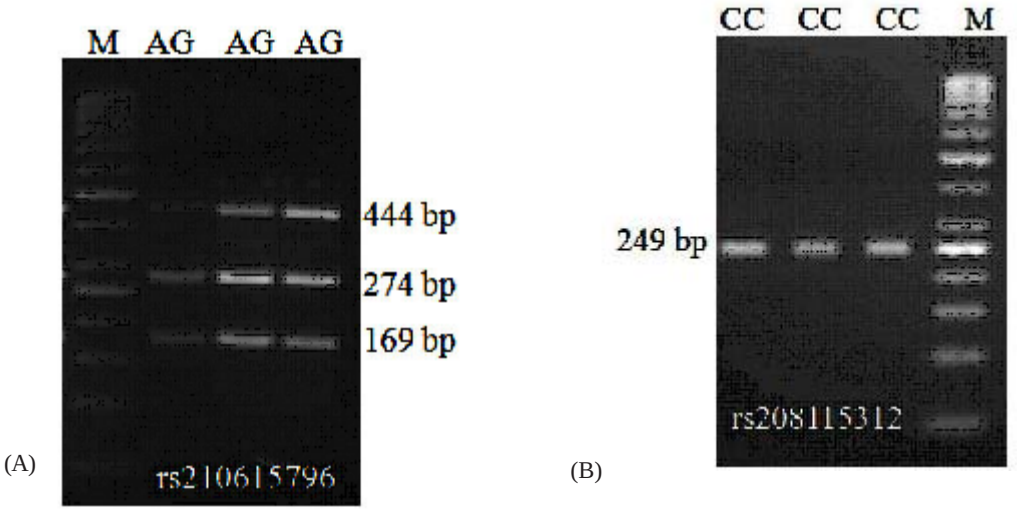

Fig. 1. PCR RFLP profile of SNP rs210615796 (A) and rs208115312 (B) resolved at 3% agarose gel. Lane M: 50 bp DNA ladder.

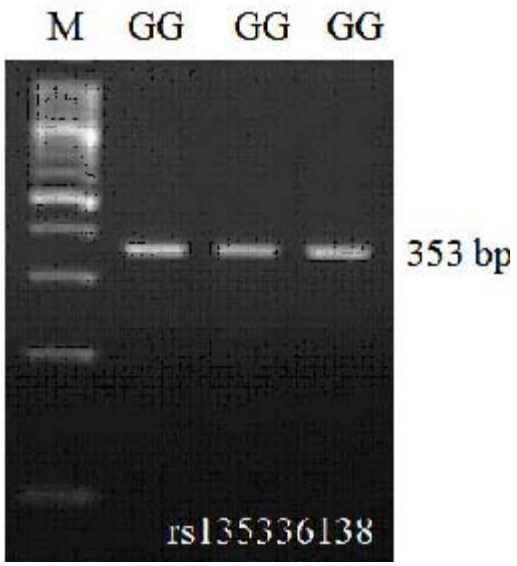

Fig. 2. PCR RFLP profile of SNP rs135336138 resolved at 3% agarose gel. Lane M: 100 bp DNA ladder

Statistical analysis (chi-square probability values) revealed that for all SNPs studied the genotype as well as allele had non-significant effect on occurrence of bovine tuberculosis (Table 3 and 4).

DISCUSSION

Despite of considerable evidence of a genetic component to TB resistance, modest effort has been directed toward identifying bovine genetic susceptibility loci. Indeed, it is only recently that effort has been directed toward quantifying the host genetic influence[15-17,25,26]. Also, European *Bos taurus* cattle appear to be more susceptible to *M. bovis* infection than *B. indicus* cattle as Ameni et al.[27] presented data from a cohort of 2500 zebus, 1900 cross breed (zebu–Holstein) and 900 Holstein cattle. Not only the prevalence of bTB higher in the Holstein population compared with the other two, but severity of pathology in skin test-positive animals was also significantly greater. Disease risks were also estimated with Holstein cattle 2.32 times more likely to be diseased than zebu cattle.

In the present study, the four SNPs analysed for association with bTB infection status were found to be non-significant in the resource population. All SNPs were non synonymous except for SNP rs42497589 (IL23R). Non synonymous SNPs alters the coded amino acid while synonymous SNPs, specifically in case of receptors, could also affect folding of protein and alter substrate specificities[28]. Regardless of the biological mechanism involved with these SNPs, any change in expression of IL12R1 and IL12RB2 could lead to alteration in the signal mediated through this receptor IL12R. Differentiation and proliferation of Th1 cells are promoted by IL12 and up regulate production of IFNG (interferon, gamma)[29]. IFNG plays major role in activation of macrophages against intracellular pathogens such

as mycobacteria in different species[30,31]. For human TB and JD SNPs in IL12RB1 are reported to be associated with resistance/susceptibility to these diseases[24,32,33]. Therefore susceptibility/resistance to bTB could be related to alteration in IL12 signalling.

The IL23 can promote indirectly a pro-inflammatory response by stimulating Th17 cells to produce IL17[34]. Several SNPs have been reported to be associated with human TB, Crohn's disease and Johne's disease which are similar in many aspect like intracellular in nature[24,35,36].

The complex genetics of bTB and the limitations implicit in the design of the present

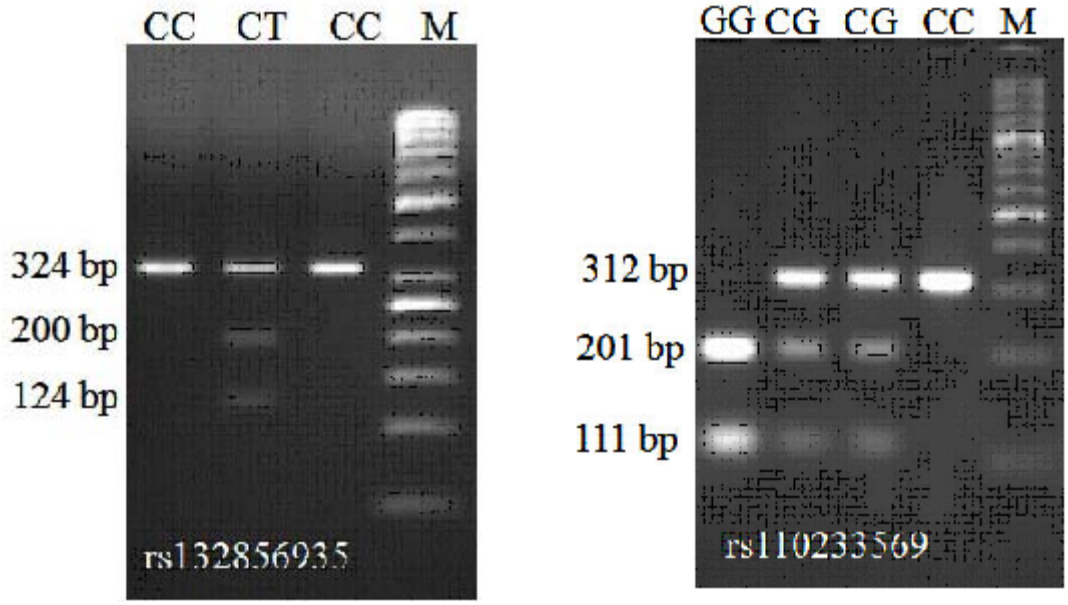

Fig. 3. PCR RFLP profile of SNP rs132856935 (A) and rs110233569 (B) resolved at 3% agarose gel. Lane M: 50 bp and 100 bp DNA ladder for (A) and (B), respectively

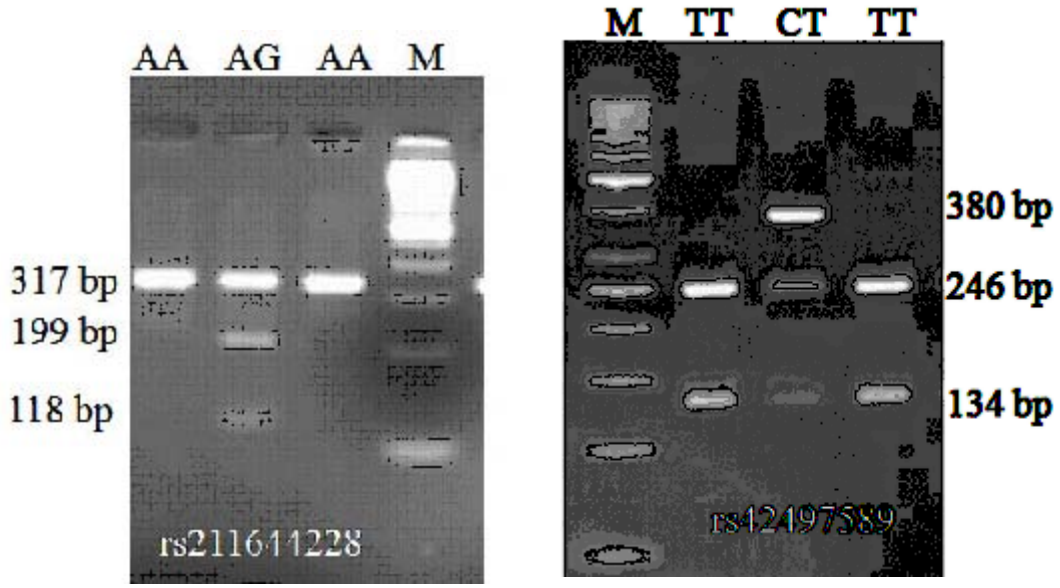

Fig. 4. PCR RFLP profile of SNP rs211644228 (A) and rs42497589 (B) resolved at 3% agarose gel. Lane M: 100 bp and 50 bp DNA ladder for (A) and (B), respectively.

experiment need to be kept in mind before drawing any conclusions from the study. The specificity and efficacy of SICCT done to develop resource population of resistant and susceptible is low. In conclusion none of the SNPs studied were found to be associated but further validation in independent, large resource population and biological characterization are warranted with more effective case-control identification. In addition other SNPs of these concerned genes could be further exploited for association studies. After proper validation the associated SNPs could be exploited, either by direct selection or making large panel of markers, to select animals more resistant to bTB. This could reduce the prevalence of bTB in dairy herds; minimize the risk to human infection and monetary losses associated with it.

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