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Single nucleotide polymorphism of *SLC11A*1, *CARD*15, *IFNG* and *TLR*2 genes and their association with *Mycobacterium avium* subspecies *paratuberculosis* infection in native Indian cattle population

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Johne's disease (JD) or paratuberculosis infection, caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is one of the major production diseases of domestic animals. There is lack of preventive and chemotherapeutic measures to control this infection. Hence, in recent years emphasis has been given to identify host genetic factors associated with resistance to infection. In the present study, we analyzed SNPs in four candidate genes (*SLC11A*1, *TLR*s, *NOD*2/*CARD* and *IFN*γ) in case and control population of native breeds of cattle. Of the four genes only SNP in *TLR*2 gene showed significant association with resistance to paratuberculosis infection. The results of the present study are promising and it is predicted that such studies will serve as guidelines in future breeding programmes.

Keywords: Host susceptibility, genetic resistance, Indian cattle, Johne's disease, paratuberculosis

Introduction

Johne's disease (JD), caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is a chronic infectious granulomatous enteritis, primarily infecting domestic ruminants leading to progressive weakness, wasting, persistent diarrhea in large ruminants, temporary and permanent loss of productivity, cachexia and death. MAP has also been associated with inflammatory bowel disease or Crohn's disease in human beings. JD is currently ranked as a one of the costliest infectious diseases of domestic ruminants. Economic losses due paratuberculosis in US dairy herds exceeds annually over $ 1.5 billion[1]. Studies in latter part of 20th century showed that JD is highly prevalent and worldwide in distribution[2]. In India, JD is endemic in farms and farmer's herds and high prevalence is reported in domestic ruminants of the country[3]. At present, no drug has been approved for the treatment of disease and antibiotic therapy where ever attempted was not successful[4]. Treatment of JD is expensive and unrewarding. Vaccination though helps in preventing disease but is not useful in eradication of disease[5]. In these circumstances, genetic selection for resistant animals can be an effective strategy for the control of this deadly and incurable disease. MAP infection heritability is estimated to be in moderate range with 0.102 being the most reliable value[6-8]. Few earlier studies have shown that considerable genetic variation exists among domestic animals in their response to MAP infection[9-10].

*SLC11A*1 gene is an iron transporter protein primarily expressed in phagosomes. This protein exhibits pleiotropic effects on the early innate macrophage response to intracellular bacterial growth including regulation of inducible nitric oxide synthase (iNOS) expression in mice[11]. Role of polymorphism in $(GT)_n$ microsatellite and SNP markers in *SLC11A*1 gene is controversial but has been discussed by many worker with some convincing results[12-14].

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*TLR*s are a family of trans-membrane signaling molecules that bind to conserved pathogen associated molecular patterns and are intrinsically involved in triggering both innate and adaptive immune response mechanisms. *TLR*s are found in all animal species. Although most mammalian species share up to eleven *TLR* genes, *TLR*1, 2 and 4 have been implicated in cellular recognition of mycobacteria, binding cell wall components including lipoproteins[15]. *TLR*2 is particularly involved in the early recognition of intestinal pathogens including mycobacteria. Allelic distribution of the *TLR*2-1903 T/C SNP was confirmed to be significantly different between infected and non-infected animals[16].

Nucleotide oligomerisation domain (NOD) proteins, such as, CARD15 are pattern recognition receptors (PRR). Ligand for CARD15 is a muramyl dipeptide derived from bacterial peptide-glycan[17] and is composed of three segments: a NH_2 terminal caspase recruitment domain, a nuclear binding domain and a LRR domain. LRR domain is capable of recognizing cell wall constituents of mycobacteria and inducing cytokine production by triggering transcription factors, such as, nuclear factor *kappa beta* (NF*kB*)[18]. A significant association between infection and variant allele was confirmed for SNP 2197T>C present in LRR domain and responsible for amino-acid substitution[13]. A significant breed effect was also reported for this SNP, with the highest proportion of the variant allele found in the Brahman-Angus population.

Interferons constitute a multi-gene family of inducible cytokines and are substances, which among other activities, induce cells to become virus resistant. A member of this group, interferon gamma (IFNγ), plays a crucial role in the innate host response to intracellular bacteria, including mycobacteria. Release of IFNγ after the initial MAP entry into the host, as part of a protective type 1-like T-cell response, has been claimed as a key factor in the control of infection and disease manifestation[19,20]. A potential association has been observed for MAP infection for the SNP 2781G/T in bovine *IFNγ* gene[13].

In the present study, we have focused on detection of already reported SNPs in the four candidate genes (*SLC11A*1, *TLR*s, *NOD*2/*CARD* & *IFNγ*) from SNP database (www.ncbi.nlm.nih.gov) and their further validation in our case and control populations. Main hypothesis was that the particular genotype/allele or haplo-type formed by a set of alleles would differ significantly in case and control populations.

Materials and Methods

Population under Study

Population under study was recruited from 2 sources. First source of 11 animals of Sahiwal breed were selected from Akha village of Bareilly district (Uttar Pradesh), India. These animals were purchased from breeding tract of Sahiwal breed in the Punjab state of India and were brought to Akha village under Sahiwal conservation project. Another source of animals was Shree Mataji Gaushal in Barsana town of Mathura district (Uttar Pradesh), India. From this gaushala (shelter for cows), 83 cattle mainly of Kosi, Sahiwal and crosses of Holstein Friesian with native (indigenous) breeds were selected. Gaushala, is a shelter for stray and abandoned cattle, therefore some of the cattle were also of non-descript type. Due to confinement and overcrowding, (dense) housing condition of cattle in gaushalas made the environment conducive for animal to animal transmission of chronic infections like MAP.

Diagnosis

All the cattle were screened by 'indigenous ELISA kit' as per the protocol of Singh *et al*[21] to establish status of MAP infection in cattle. In ELISA, S/P ratio of >0.4 was considered positive and <0.25 was considered as negative[22]. While cattle showing S/P ratio between 0.25 and 0.4 was low positive and was considered inconclusive and excluded from the study. Of 94 selected animals, 47 were cases and rest 47 was control animals.

DNA Isolation

DNA from blood of population under study was extracted by phenol:chloroform extraction method as described by Singh *et al*[13]. Quality of DNA was checked by 1% agarose gel electrophoresis and concentration was measured by Qubit® fluorometer. Samples with DNA concentration above 50 ng/µL were taken for study.

Source Panel of SNPs

SNPs selected for the present investigation were already identified but their association with MAP infection was not known. SNPs were selected from SNP database of NCBI (www.ncbi.nlm.nih.gov). All SNPs selected were assayable through PCR-RFLP.

Five previously reported SNPs in the gene *SLC11A*1 were selected from various exonic regions; one from exon 2 (rs109614179), two from exon 3 (rs110905610 & rs110514940), one from exon 8 (rs109915208), and

one from exon 11 (rs109453173). One SNP each in exon 4 of *IFNγ* gene (rs110853455) and single exon of *TLR*2 genes were selected. Further, two SNPs were selected from gene *NOD2/CARD15*; rs111009394 from exon 3 and rs110536091 from intronic region between exon 9 and 10.

Allele Determination

Alleles at all the selected SNPs were determined by PCR-RFLP technique. PCR primers for different SNPs, their annealing temperatures and amplicon size are depicted in Table 1; while restriction enzymes and digestion conditions with RE fragment sizes are given in Table 2. General conditions for PCR amplification were 94°C for 2 min, 35 cycles consisting of denaturation at 94°C for 45 sec, annealing at varying temperature (Table 1) for 45 sec and extension at 72°C for 30 sec, followed by final extension at 72°C for 5 min. PCR products were checked by 2% agarose gel electrophoresis and further processed by RE digestion with their respective enzymes (Table 2). RE digestion products were resolved by electrophoresis on 3.5% Metaphore agarose gel.

Statistical Analysis

The univariate logistic regression analysis was used by considering the infection status as categorical response variable (yes/no) and SNPs as possible

Table 1—PCR primers and amplification conditions for the analysis of alleles of SNPs

Primers	Region	SNP db	Orientation	Sequence (5'-3')	Annealing temp (°C)	Amplicon size (bp)
SLC-1	SLC11A1	rs109614179	Sense	CAGAAAGAGGCAGGTCACTCA	60.5	226
			Antisense	ATCCGGTGCATCCTGAAAGA		
SLC-2a	SLC11A1	rs110905610	Sense	ACCCAAGACCCCTCATTCCA	60.5	207
			Antisense	CACCCACTTAGCCTGGTCAC		
SLC-2b	SLC11A1	rs110514940	Sense	ACCCAAGACCCCTCATTCCA	60.5	207
			Antisense	CACCCACTTAGCCTGGTCAC		
SLC-10	SLC11A1	rs109915208	Sense	TCACTCACCCCACAGTACG	53	151
			Antisense	GGTAGATGTTGTGGGGCATGA		
SLC-15	SLC11A1	rs109453173	Sense	CTGACCACACCCCTCCTTC	60.5	191
			Antisense	TCCTGAGTGGTCTGCCTTGT		
IFNγ-3	IFNγ	rs110853455	Sense	TTGGGCCTAGACAGCAGAAT	59.5	665
			Antisense	TTTTGTCAGGGCACAAGTCA		
NOD-2	NOD2	rs111009394	Sense	TTCTCTTGGCTTCCTGGTGC	54	815
			Antisense	TCCTGGCTCCCAGCATAAAG		
NOD-5	NOD2	rs110536091	Sense	CTGGTCCAGTCCTCACTCAA	53	166
			Antisense	AATGGCCTCCTCCCAAACAC		
TLR	TLR2	rs55617172	Sense	TTAAACTCCATCCCCTCTGG	59.2	245
			Antisense	TAAAGGGACCTGAACCAGG		

Table 2—Restriction enzymes and restriction conditions for analysis of SNPs

Region	SNP db	Restriction enzyme	Incubation temp (°C)	Amplicon size (bp)	1st homozygous	Heterozygous	2nd homozygous
SLC11A1	rs109614179	HhaI	37	226	226 (AA)	226, 126, 100 (AG)	126, 100 (GG)
SLC11A1	rs110905610	Hpych4v	37	207	207 (AA)	207, 37, 170 (AG)	37, 170 (GG)
SLC11A1	rs110514940	AciI	37	207	48, 159 (CC)	207, 48, 159 (CA)	207 (TT)
SLC11A1	rs109915208	Bpu10I	37	151	35, 116 (CC)	151, 35, 116 (CT)	151 (TT)
SLC11A1	rs109453173	BpmI	37	191	36, 155 (CC)	191, 36, 155 (CG)	191 (GG)
IFNG	rs110853455	HphI	37	665	129, 536 (AA)	129, 536, 665 (AG)	665 (GG)
NOD-2	rs111009394	BsaHI	37	815	705, 110 (CC)	815, 705, 110 (CT)	815 (TT)
NOD-2	rs110536091	HpyaV	37	166	50, 116 (CC)	50, 116, 166 (CT)	166 (TT)
TLR-2	rs55617172	EcoRV	37	245	245 (CC)	245, 182, 63 (CA)	182, 63 (AA)

explanatory variables. Data were analyzed using PROC LOGISTIC procedure of SAS 9.3 and ODDs ratios (ORs) with 95% CIs were calculated. The relative risk of incidence among the genotypes was analyzed using a univariate logistic regression model. The PROC ALLELE procedure was used for testing of Hardy-Weinberg (H-W) equilibrium, estimation of heterozygosity and polymorphism information content (PIC) of SNPs markers used in present investigation.

Results

Of the total population screened, 94 animals were selected in 2 groups, 47 cases and 47 controls, based on results of ELISA using criteria explained above. Results of univariate logistic regression analysis revealed no significant effect of breed on disease occurrence.

SLC11A1 Gene

Of 5 SNPs selected from this gene, 2 gave monomorphic pattern. For the rest 3, frequencies of common alleles for SNP-1, -2 and -3 were 0.68, 1 and 0.71, respectively in case and 0.65, 0.99 and 0.72, respectively in control population (Tables 3 & 4). Similarly, the frequencies of rarer alleles for SNP-1, -2 and -3 were 0.32, 0 and 0.29, respectively in case and 0.35, 0.01 and 0.28, respectively in control group. None of these frequencies differ significantly between two groups and failed to genetically distinguish infected animals from healthy animals. Linkage

Table 3—Distribution of allelic frequency in case and control animals

SNPs	Gene	Allele	Allele frequency		p-value	Odds ratio (95% CI)
			Case	Control		
rs109614179	SLC11A1	G	64 (0.68)	61 (0.65)	0.64	1.00
(SNP-1)		A	30 (0.32)	33 (0.35)		0.86 (0.47-1.58)
rs109915208	SLC11A1	T	0 (0)	1 (0.01)	0.31	1.00
(SNP-2)		C	94 (1)	93 (0.99)		>999.99(0.001- >999.99)
rs109453173	SLC11A1	G	67 (0.71)	68 (0.72)	0.87	1.00
(SNP-3)		C	27 (0.29)	26 (0.28)		1.05 (0.55- 1.99)
rs111009394	CARD15	T	59 (0.63)	51 (0.54)	0.23	1.00
(SNP-4)		C	35 (0.37)	43 (0.46)		0.70 (0.39- 1.26)
rs110536091	CARD15	T	66 (0.7)	62 (0.66)	0.53	1.00
(SNP-5)		C	28 (0.3)	32 (0.34)		0.82 (0.44-1.52)
rs110853455	IFNG	G	82 (0.88)	82 (0.88)	1.00	1.00
(SNP-6)		A	12 (0.12)	12 (0.12)		1.00 (0.42- 2.35)
rs55617172	TLR2	C	76 (0.8)	64 (0.68)	0.04	1.00
(SNP-7)		A	18 (0.2)	30 (0.32)		0.50 (0.25- 0.99)

Table 4—Distribution of genotype frequency in case and control animals

SNP	Gene	Genotype	Genotype frequency		p-value	Odds ratio (95% CI)
			Case	Control		
rs109614179	SLC11A1	GG	24 (0.51)	18 (0.38)	0.16	1.00
(SNP-1)		AA	7 (0.15)	4 (0.09)		1.3 (0.33-5.17)
		AG	16 (0.34)	25 (0.53)		0.48 (0.20-1.15)
rs109915208	SLC11A1	CT	0 (0)	1 (0.02)	0.31	1.00
(SNP-2)		CC	47 (1)	46 (0.98)		>999.99(0.001- >999.99)
rs109453173	SLC11A1	GG	25 (0.53)	23 (0.49)	0.37	1.00
(SNP-3)		CC	5 (0.11)	2 (0.04)		2.3 (0.4-13.03)
		CG	17 (0.36)	22 (0.47)		0.71 (0.3-1.66)
rs111009394	CARD15	TT	20 (0.43)	14 (0.3)	0.43	1.00
(SNP-4)		CC	8 (0.17)	10 (0.21)		0.56 (0.17-1.77)
		CT	19 (0.4)	23 (0.49)		0.58 (0.23-1.44)
rs110536091	CARD15	TT	19 (0.4)	15 (0.32)	0.39	1.00
(SNP-5)		CT	28 (0.6)	32 (0.68)		0.69 (0.29-1.61)
rs110853455	IFNγ	GG	36 (0.77)	35 (0.74)	0.55	1.00
(SNP-6)		AA	1 (0.02)	0 (0)		>999.99(0.001- >999.99)
		AG	10 (0.21)	12 (0.26)		0.81 (0.31-2.11)
rs55617172	TLR2	CC	31 (0.66)	27 (0.57)	0.04	1.00
(SNP-7)		AA	2 (0.04)	10 (0.21)		0.17 (0.03-0.86)
		AC	14 (0.3)	10 (0.21)		1.22 (0.46-3.19)

disequilibrium studies in different possible combinations revealed that the SNP-1 is linked ($p \leq 0.05$) with both SNP-2 and SNP-3, but SNP-2 and SNP-3 were not linked together ($p > 0.05$) and segregating independently. As there is no significant association with disease occurrence in any of loci, so haplotype formed by linked loci will not be fruitful. All the three loci were found to be in H-W equilibrium.

***CARD15* Gene**

Allele frequency among both the SNPs studied was not differing significantly in this gene also (Tables 3 & 4). Frequencies of major alleles among case population were 0.63 and 0.7 in SNP-4 and -5, respectively. Not much difference in control population was found in comparison to case and frequencies were 0.66 and 0.54, respectively. Both the loci were completely linked but this haplotype has no significant effect on disease occurrence. SNP-4 was in H-W equilibrium, but distribution of SNP-5 did not fit in H-W equilibrium in our population.

***IFNγ* Gene**

For single SNP studied, no difference was observed in allele frequency between case and control population. For both the groups, allele frequencies were 0.88 and 0.12 for common and rarer alleles, respectively (Tables 3 & 4). As expected, locus was found to be in H-W equilibrium.

***TLR2* Gene**

This was the only gene in the present study where both allelic and genotype frequencies differed significantly between case and control, representing association of this gene with paratuberuclosis infection. Estimated ORs for this locus (SNP-7) revealed the relative resistance of AA genotype with respect to both of the genotypes (Tables 3 & 4). This locus also departed significantly from H-W equilibrium.

Discussion

Presently, only few studies have explored the association between paratuberculosis susceptibility and candidate genes. Majority of them have not succeeded in finding strong associations[23-24], likely due to limitation in sample size and sensitivity of the diagnostic tests used. In the present study, four candidate genes were selected and, of the four, only *TLR*2 gene had shown a significant effect ($p \leq 0.05$) on occurrence of paratuberculosis with respect to gene and genotype frequency of case and control population. Rest of the three genes failed to distinguish between infected and healthy animals.

SNP-1, -2 and -3 of *SLC11A*1 gene failed to exhibit any association with disease resistance, so our results are inconsistent with findings of several other workers, who investigated the role of *SLC11A*1 gene not only in paratuberculosis[13,14,25] but also in other related disease like tuberculosis[26] and IBD/CD[27]. SNPc.1067C>G of *SLC11A*1 gene found to be a causal variant that causes an amino acid change in codon 356 from proline to alanine (P356A) that could alter SLC11A1 protein function[14]. Similarly, our results for other 2 genes are also inconsistent with several workers. Pinedo *et al*[13] reported a strong association between different allelic variants of *IFNγ* and infection status of paratuberculosis. Possible reason for this discrepancy may be the less genetic variability observed for the locus studied in our population as clearly visible by the low frequency of A allele and high frequency of G allele, which is drifting toward fixation.

In the present study, *TLR*2 was the only gene found to be associated with resistance/susceptibility to paratuberculosis (Tables 3 & 4). Odd ratios of disease occurrence for AA genotype were only 0.17 as compared with reference genotype CC. Here one more interesting point needs to be emphasized that animals with AC genotype, *i.e.*, heterozygotes, were more abundant in case compared to control. Despite the odd ratios of A allele being lower than C allele, the odd ratios of AC were 1.22 times than the odd ratios of CC genotype. It is a possible example of heterozygous disadvantage, but it requires further confirmation.

If we want to confer resistance to MAP infection in our population, then a selection programme favouring AA genotype may be fruitful but attention should be given on identification of heterozygous animals as heterozygous are more susceptible to disease than any of the homozygotes. Consequently, selection will favour fixation of A allele in the population. Despite the strong association of A allele with MAP resistance, the C allele was found more in our population. This can be attributed to two major reasons. First that our sample is being collected from 2 different sources and even in gaushala, a large number of animals are being continuously added in the herd for shelter purpose. So our population is largely is of emigrated animals and it may not be in equilibrium. Second is consideration of the fact that

the same locus may affect several other traits also or the heterozygous genotype may also be positively affecting any other trait. So a combined selection for many traits simultaneously in the nature may be leading to an equilibrium frequency, which favours more of C alleles or heterozygous animals. A recent study by Koets *et al*[16] revealed the similar findings with *TLR*2-1903 T/C SNP where CC and CT genotypes were at 1.7 times greater risk compared to TT genotype for getting MAP infection.

Conclusion

The present analysis suggests a potential connection between polymorphisms in *TLR*2 gene and the risk of paratuberculosis infection. Further validation on a large-scale study may yield some significant and conclusive results.

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