



An association study of SNPs with susceptibility to bovine paratuberculosis infection in cattle

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Received: 11 December 2013; Accepted: 18 January 2014

ABSTRACT

The study was conducted to identify SNPs of quantitative trait loci (QTL) associated with susceptibility to *Mycobacterium avium* ssp. *paratuberculosis* (MAP) infection in cattle. Total 20 SNPs from the cattle QTL database were selected on the basis of the potential role in *Mycobacterium* susceptibility and a case: control association study was conducted in cattle. Out of 20 SNPs total 17 SNPs were polymorphic and 3 were monomorphic in population. The SNP (rs41945014) was significantly associated with MAP and revealed that ODDs of GG and GT genotypes versus TT genotype were 1.22 (0.33–4.49; 95% CI) and 3.37 (1.23–9.23; 95% CI), respectively. The proportion of GG and GT genotypes were significantly higher in bovine paratuberculosis positive animals suggested that selection against these 2 genotypes may confer resistance against bovine paratuberculosis. The all other 16 polymorphic SNPs of the present investigation were not differing significantly in case-control animals.

Key word: Cattle, MAP, Paratuberculosis, Quantitative trait loci, SNP

The bovine paratuberculosis (PTB), a contagious fatal disease also known as Johne's disease, is caused by an acid fast bacteria *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Paratuberculosis is now-a-days viewed as one of the most important and wide spread bacterial diseases of ruminants. Cattle less than 6 months of age are most susceptible, and it was estimated that approximately one-third of calves may develop infection with a single exposure. In India, paratuberculosis is in endemic form (Tripathi *et al.* 2007) and it has zoonotic potential. High prevalence of paratuberculosis was reported in domestic livestock using indigenous, sensitive and MAP specific test in north India. Sero-prevalence of bovines PTB was 29.0% (28.6% in buffalo and 29.8% in cattle) in north India. In Uttar Pradesh and Punjab, seroprevalence of bovine PTB was 32.9 % (31.9% in buffalo and 37.6% in cattle) and 25 % (23.3% in buffalo and 26.9% in cattle), respectively. Susceptibility to MAP infection is heritable

with heritability estimates ranging from 0.06 to 0.102 (Gonda *et al.* 2006). These studies provided evidence for the existence of important genetic variation in susceptibility to paratuberculosis. Some reports are available about association study of bovine paratuberculosis with candidate gene polymorphism (Ruiz *et al.* 2007, Settle *et al.* 2009, Pinedo *et al.* 2009, Pant *et al.* 2010, Koets *et al.* 2010). But till date in India no report is available on candidate gene polymorphism with paratuberculosis infection in cattle. Keeping in view above facts the present study was proposed by selecting 20 SNPs for finding their association with MAP infection in cattle.

MATERIALS AND METHODS

A case-control resource panel for bovine paratuberculosis was developed at the animal genetics division under the divisional project —Genetic Predisposition to host resistance against Bovine Paratuberculosis in cattle— to develop this resource panel more than 300 cattle's were screened for presence of bovine paratuberculosis. The Johnin PPD test and unabsorbed indirect ELISA were used for diagnosis of cattle infected with paratuberculosis. Bovine paratuberculosis infection was screened by using Johnin PPD test. The test involved injecting 0.1 ml of Johnin PPD intradermal into cervical region of the animal and the subsequently detection of swelling (delayed hypersensitivity) at the site of injection after 72 h. The

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reaction is commonly considered to be negative if swelling is observed; not more than 2 mm; if the increase in skin-fold thickness is more than 2 mm and less than 4 mm the reaction is considered to be inconclusive. The reaction is considered to be positive if there is an increase of 4 mm or more in skin-fold thickness.

Bovine paratuberculosis infection was used to screen by unabsorbed indirect ELISA. The ELISA with an S/P ratio of >0.4 were considered positive and <0.25 were considered as negative. While animals showing S/P ratio between 0.25 and 0.4 were low positive and were considered inconclusive.

On the basis of the above mentioned tests total 44 paratuberculosis affected and 49 healthy control animals were used in the present investigation.

Blood collection and DNA isolation: About 5 ml of venous blood was collected under sterile condition, from the jugular vein of the animal into a sterile 15 ml polypropylene centrifuge tube containing EDTA (1.8mg EDTA / milliliter of blood). The samples were kept in deep freeze at -20°C till the isolation of DNA. DNA from the whole blood was isolated using genomic DNA extraction kit and kept at -20°C .

Source panel of SNPs, PCR-RFLP and gel electrophoresis: From animal QTL database (<http://www.animalgenome.org/cgi-bin/QTLdb>), the QTLs associated with bovine paratuberculosis susceptibility/tolerance were searched and 20 promising QTLs (rs109640417, rs43070062, rs42279934, rs110002076, rs42396640, rs109059425, rs41945014, rs41962859, rs29012843, rs29012842, rs41924236, rs41579048, rs41663541, rs110898024, rs110705351, rs109621404, rs109288717, rs110490432, rs41703350, rs110383586), assessable through PCR-RFLP were identified. The primers targeting these QTL were designed. PCR-RFLP technique was used to identify the SNPs in the QTLs. The RE digested PCR product was electrophoresed in 3% to 5%w/v agarose gel (low EEO) for 2 h at 60 volt. About 23 μl of digested product mix with 2 μl of 6 $\times$ loading dye were loaded into each well along with 100 bp DNA ladder in a separate lane. After completion of gel electrophoresis the digested product were visualized by UV transilluminator and documented to detect the genotype of each sample.

Statistical analysis: The univariate logistic regression analysis was used by considering the infection status as categorical response variable (yes/no) and SNPs as possible 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 EXACT analysis was used for continuity correction for SNPs association where cells were having less than 5 observations. Initially in univariate logistic regression analysis, the non-genetic factors like age (2 level), sex (2 level) and breed (2 level) were fitted and found that none of these effects were significantly associated with incidences of bovine paratuberculosis. Hence, further these non genetic factors were dropped out

from logistic models for studying effect of SNPs on bovine paratuberculosis. The PROC ALLELE procedure was used for testing of HWE, estimation of heterozygosity, polymorphism information content (PIC) and linkage disequilibrium (LD) of SNPs markers used in present investigation.

RESULTS AND DISCUSSION

The case: Control population was genotyped for 20 SNPs by PCR-RFLP. For, 3 SNPs namely rs3070062, rs42396640 and rs41663541 showed monomorphic pattern and suggested for the lack of polymorphism in our resource population. In remaining 17 SNPs, rs109640417, rs42279934, rs41924236, rs109621404 and rs110490432 showed only 2 genotypes and other 12 SNPs revealed 3 possible genotypes. The heterozygosity, polymorphic information content and allelic diversity was estimated at 17 polymorphic SNPs loci. Heterozygosity varied widely from a very low 4.30 % for rs42279934 to a high estimate of 83.87 % for rs41924236. The PIC ranged from a very low estimate of 4.12 at rs42279934 to 37.50 % at rs109288717 locus revealing a low to moderate polymorphism at these SNPs. Allelic diversity estimates also revealed similar trend and ranged from 4.2 % at rs42279934 to 50.00 % at rs109288717. The 5 SNPs of present investigation departed significantly from Hardy Weinberg Equilibrium (HWE) and all other SNPs were in HWE. The linkage disequilibrium (LD) was estimated and the rs109640417 was found linked significantly ($P \leq 0.05$) with rs41962859, rs29012842, rs41579048, rs109621404 and rs110490432; the rs110002076 was linked with rs29012842 and rs110898024; the rs109059425 was linked with rs41945014 and rs110490432; the rs29012842 was linked with rs110490432; rs41579048 was linked with rs109621404. All the other paired combinations ($^{17}\text{C}_2$) were not linked significantly with each other. The only significant

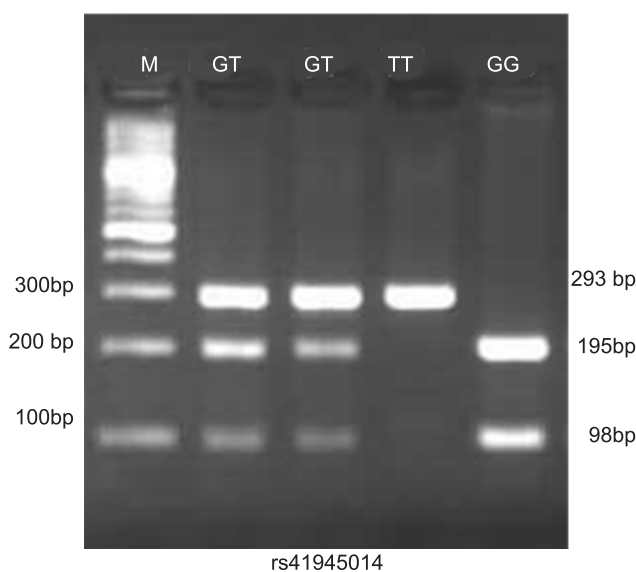


Fig. 1. PCR-RFLP profile of SNP rs41945014 in positive and negative animal. M= 100bp.

SNP (rs41945014) of present study was not found linked with any other SNPs, and was found to be in HWE and thus possibility of haplotype study was ruled out.

The genotypic frequencies at all 17 SNP locus are given in Table 1. The frequencies of different genotypes ranged from 0.023 to 0.977 in case and from 0.00 to 0.939 in control population. The univariate regression analysis revealed that the genotypic frequency at rs419445014 associated significantly ($P=0.033$) with paratuberculosis and all other 16 SNPs showed nonsignificant ($P\leq 0.05$) differences for the genotypic frequencies in case and control population

(Table 1). At this rs419445014, three genotype TT (294bp), GG (196bp, 98bp) and GT (294bp, 196bp, 98bp) were detected (Fig. 1). The genotypic frequencies of TT, GG and GT were 0.182, 0.136 and 0.682 in MAP infected cattle, whereas these frequencies were 0.367, 0.224 and 0.408, respectively in control population. The differences for the genotypic frequencies between case and control population were found to be statistically significant ($P=0.033$). The ODDs of GG and GT genotypes vs TT genotypes were 1.22 (95%CI: 0.33–4.49) and 3.37 (95% CI: 1.23–9.23), respectively, suggesting that GG and GT genotypes are more

Table 1. Genotypic frequency distribution and their association with MAP

SNP	Genotype	Genotype frequency		p-value	Odds ratio (95% CI)
		Case	Control		
rs109640417	CC	36(0.8182)	42(0.8571)	0.610	1.00
	AC	8(0.1818)	7(0.1429)		1.33(0.44 - 4.03)
rs42279934	GG	43(0.9773)	46(0.9388)	0.619	1.00
	GC	1(0.0227)	3(0.0612)		0.35(0.03– 3.56)
rs110002076	GG	25(0.5682)	22(0.4490)	0.525	1.00
	AA	4(0.0909)	6(0.1224)		0.58(0.14– 2.35)
	AG	15(0.3409)	21(0.4286)		0.62(0.26– 1.50)
rs109059425	GG	35(0.7955)	40(0.8163)	1.00	1.00
	AA	3(0.0682)	3(0.0612)		1.14(0.21– 6.03)
	AG	6(0.1364)	6(0.1224)		1.14(0.33– 3.86)
rs41945014	TT	8(0.1818)	18(0.3673)	0.033	1.00
	GG	6(0.1364)	11(0.2245)		1.22(0.33– 4.49)
	GT	30(0.6818)	20(0.4082)		3.37(1.23–9.23)
rs41962859	TT	5(0.1136)	5(0.1020)	0.403	1.00
	GG	13(0.2955)	21(0.4286)		0.61(0.15–2.56)
	GT	26(0.5909)	23(0.4694)		1.13(0.29– 4.40)
rs29012843	TT	18(0.4091)	22(0.4490)	0.299	1.00
	CC	6(0.1364)	2(0.0408)		3.66(0.65–20.42)
	CT	20(0.4545)	25(0.5102)		0.97(0.41–2.30)
rs29012842	CC	7(0.1591)	13(0.2653)	0.195	1.00
	AA	17(0.3864)	22(0.4490)		1.43(0.47–4.37)
	AC	20(0.4545)	14(0.2857)		2.65(0.84–8.33)
rs41924236	AG	36(0.8182)	42(0.8571)	0.610	1.00
	AA	8(0.1818)	7(0.1429)		1.33(0.44–4.03)
rs41579048	TT	3(0.0682)	6(0.1224)	0.691	1.00
	CC	29(0.6591)	32(0.6531)		1.81(0.41–7.91)
	CT	12(0.2727)	11(0.2245)		2.18(0.43–10.90)
rs110898024	GG	25(0.5682)	26(0.5306)	0.695	1.00
	AA	2(0.0455)	1(0.0204)		2.08(0.17–24.40)
	AG	17(0.3864)	22(0.4490)		0.80(0.34–1.85)
rs110705351	GG	23(0.5227)	29(0.5918)	0.597	1.00
	AA	1(0.0227)	0(0)		>999.99(0.001–>999.99)
	AG	20(0.4545)	20(0.4082)		1.26(0.55–2.88)
rs109621404	AG	12(0.2727)	8(0.1633)	0.199	1.00
	AA	32(0.7273)	41(0.8367)		0.52(0.19–1.42)
rs109288717	TT	11(0.2500)	11(0.2245)	0.785	1.00
	CC	9(0.2045)	13(0.2653)		0.69(0.21–2.28)
	CT	24(0.5455)	25(0.5102)		0.96(0.35–2.62)
rs110490432	GG	36(0.8182)	40(0.8163)	0.981	1.00
	AG	8(0.1818)	9(0.1837)		0.98(0.34–2.83)
rs41703350	TT	34(0.7727)	40(0.8163)	0.602	1.00
	CT	10(0.2273)	9(0.1837)		1.30(0.47–3.58)
rs110383586	TT	22(0.5000)	21(0.4286)	0.791	1.00
	CC	4(0.0909)	5(0.1020)		0.76(0.18–3.23)
	CT	18(0.4091)	23(0.4694)		0.74(0.31–1.76)

susceptible to MAP infection in comparison to TT genotype.

All the 17 SNPs taken from the QTLs had significant effect on susceptibility to MAP infection (Settle *et al.* 2009, Kirkpatrick 2010) based on the genotyping using the Illumina BovineSNP50 Beadchip. But we found the significant (rs419445014) association of only 1 SNP with MAP infection in our resource population. It is further required to validate these SNPs on comparatively larger population with more precise/accurate diagnostic procedures. Ruiz *et al.* (2007) reported that SNP N23 of Nramp1 (located in the BTA2 and comprises 15 exons) was genetically associated with resistance to the paratuberculosis infection ($P=0.0478$). Koets *et al.* (2010) reported that the TLR2-1903 T/C SNP was significantly associated with resistance to MAP where cows with CT and CC genotypes were at 1.7 (95% CI: 1.2, 2.8) times the odds of being MAP-infected compared to cows with the TT genotype. Pant *et al.* (2010) reported 22 SNPs on 7 different chromosomes significantly associated with the disease trait using this genome-wide threshold. Pinedo *et al.* (2009) characterized the distribution of polymorphisms in the bovine CARD15 gene and test their association with paratuberculosis infection in cattle. Pinedo *et al.* (2009) reported a significant association between infection status and BoIFNG-SNP12781 and SLC11A1-275-279-281 microsatellites. Verschoor *et al.* (2010) reported that SNPs in *IL10RA* are associated with MAP infection status in dairy cattle. Pant *et al.* (2011) reported that 4 SNPs, viz. IFNGR2, IL12RB1, IL12RB2, and IL23R were found to be associated with the MAP infection status of the resource population.

Taken altogether, these results indicated that the SNPs at rs419445014 locus would be valuable for genotyping the animals for selection against *Paratuberculosis* infection in cattle. The rs419445014 was present at BTA20 and no other candidate gene was representing through this SNPs. Also further investigation on larger population would warrant about strength of association of investigated SNPs.

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