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RESEARCH ARTICLE

Evaluation of the association of *SLC11A1* gene polymorphism with incidence of paratuberculosis in goats

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Abstract. Paratuberculosis is one of the chronic granulomatous enteritis that predominantly affects ruminants worldwide, caused by *Mycobacterium avium* ssp. *paratuberculosis* (MAP). In ruminants, microsatellite polymorphisms of the 3′ untranslated region (3′UTR) of the solute carrier family 11 member A1 (*SLC11A1*) gene were associated with resistance to intracellular pathogen infections. This research was carried out to detect the polymorphisms in A and B regions of the 3′UTR of *SLC11A1* gene and to evaluate the potential association between these polymorphisms and MAP infection in goats. MAP-specific antibodies were detected by ELISA and MAP infection was confirmed by IS900 PCR in 150 adult goats from different regions of Kerala, India. The polymorphism of microsatellite regions A and B at 3′UTR of the *SLC11A1* gene was analysed in goats by an automated technique, fragment analysis, using fluorescent-tagged forward primers. Eight alleles with sizes ranging from 221 to 239 bp were found in region A. Region B revealed two alleles, 117 bp (B_7) and 119 bp (B_8). Animals with B_8 alleles were found to have higher incidence of paratuberculosis than animals with B_7 alleles ($P < 0.01$). There was no statistically significant association found between region A genotypes and paratuberculosis incidence. These results suggest that caprine *SLC11A1* gene has significant role in paratuberculosis resistance in goats and further studies might help in development of a PCR-based genotyping test for paratuberculosis resistance and selection of superior animals for future goat breeding programmes.

Keywords. paratuberculosis; microsatellite; natural resistance; *SLC11A1* gene; goats.

Introduction

Paratuberculosis or Johne's disease is considered as one of the most serious, contagious, bacterial diseases of ruminants, especially in cattle, sheep and goats, caused by *Mycobacterium avium* ssp. *paratuberculosis* (MAP). It is characterized by diarrhoea, rapid weight loss, reduced milk production, reproductive failure and death in farm animals (Chiodini *et al.* 1984). Infections with MAP in caprine herds result in significant economic loss, through slow progressive wasting and the subsequent death of the infected animals. A study conducted by Singh *et al.* (2008a, b) showed that prevalence of MAP in domestic livestock in India was moderately higher and there is urgent need to control the disease at national level to improve per animal productivity in India. The disease is zoonotically important, since IS900 characterization of positive cultures in stool and biopsies from confirmed cases of Crohn's disease in northern India, proved the association between MAP and Crohn's disease (Singh *et al.* 2008a, b). Diagnostic tests include isolation of MAP, Ziehl–Nielsen's (ZN) acid fast staining, enzyme-linked immunosorbent assay (ELISA) and IS900 PCR. Since these tests are time consuming and costly, the early detection of subclinical paratuberculosis is difficult, combined with the incapability of currently available vaccines to prevent the disease or disease shedding, necessitates the adoption of newer techniques for the prevention of MAP

infection. This could be overcome by selection of disease-resistant animals by appropriate selection methods such as marker-assisted selection (MAS). Most of the recent researches regarding disease resistance suggest that limiting the spread of disease may be possible through selective breeding of animals based on genetic markers associated with resistance or susceptibility. One of the genes that have been targeted for this purpose is the solute carrier family 11 member A1 (*SLC11A1*) (Bellamy *et al.* 1998) in goats, which is located in chromosome 2 (Vacca *et al.* 2011).

The *SLC11A1* is a potential candidate gene that confers innate resistance against various intracellular pathogens including MAP. The *SLC11A1* gene, previously known as natural resistance-associated macrophage protein 1 (*NRAMP1*) gene is a member of large family of metal ion-transport proteins. *SLC11A1* gene encodes SLC11A1 protein which is a member of large family of metal ion-transport proteins linked to infectious disease susceptibility in mouse (Vidal *et al.* 1993), functions as a pH-dependent transporter that prevents the acquisition of divalent cations like Fe^{2+} and Mn^{2+} towards the cytosol through the phagosome membranes and thus, it favours bacterial killing (Forbes and Gros 2003). *SLC11A1* gene delivers bivalent metal cations from the cytosol into acidic endosomal and lysosomal compartments under normal physiological conditions, where the Fenton and Haber–Weiss reactions generate toxic antimicrobial radicals for direct antimicrobial activity against phagocytosed microorganisms (Goswami *et al.* 2001). This gene has pleiotropic effects on macrophage function that include increased keratinocyte chemoattractant (chemokine KC), tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), inducible nitric oxide synthase and major histocompatibility (MHC) class II expression; all of them are important in resistance to intracellular pathogens (Awomoyi 2007).

In ruminants, microsatellite polymorphisms of 3′ untranslated region (3′UTR) of the *SLC11A1* gene were associated with resistance to *Brucella abortus*, *Mycobacterium bovis* and *Mycobacterium avium* ssp. *paratuberculosis* (Barthel *et al.* 2001; Reddacliff *et al.* 2005; Borriello *et al.* 2006; Capparelli *et al.* 2007a; Martínez *et al.* 2008; Pinedo *et al.* 2009; Korou *et al.* 2010; Kadarmideen *et al.* 2011; Taka *et al.* 2013, 2015). There are two polymorphic microsatellites in the 3′UTR of the caprine *SLC11A1* gene with a variation in the number of guanine thymine repeats $(GT)_n$ (Vacca *et al.* 2011). The region A was found to be more polymorphic than the region B, where only two alleles were reported for region B in goats (Korou *et al.* 2010; Vacca *et al.* 2011). Thomas and Joseph (2012) reviewed the role of *SLC11A1* gene in diseases resistance especially for intracellular pathogens and opined that disease is the most important constraint in the animal production system and the selection of animals for increased genetic resistance to diseases will lead to the production of a healthy and productive stock. The present study was designed to detect the polymorphisms in A and B regions of the 3′UTR of *SLC11A1* gene and to evaluate the potential association between these polymorphisms and the presence or absence of MAP infection in goats of Kerala, south India.

Materials and methods

Sample collection

All experimental procedures were performed according to the guidelines of the Institutional Animal Ethics Committee of Kerala Veterinary and Animal Sciences University. Blood, serum and faecal samples were collected from 150 adult goats comprising 50 Malabari, 47 Attappady Black and 53 Malabari crossbreds from Thrissur and Malappuram districts of Kerala, India. Animals included in the present study were maintained at similar environmental conditions and not vaccinated for paratuberculosis. Blood samples were used for the isolation of genomic DNA for microsatellite genotyping of the goats under study, whereas serum and faecal samples were used for the detection of MAP antibodies by ELISA and MAP antigens by IS900 PCR.

Genomic DNA isolation and genotyping

Genomic DNA was extracted from whole blood using the standard phenol–chloroform extraction procedure. DNA concentration of samples was quantified by Nano Drop (NanoDrop, ThermoScientific, USA) and stored at −20°C until used. PCR was carried out to amplify both A and B regions at the 3′UTR of the caprine *SLC11A1* gene. Primer pairs for A region (Ex15F1: 5′-GTCTGGACCTGTCTCATCACC-3′ and Ex15R1: 5′-ACTCCCTCTCCATCTTGCTG-3′), and B region (Ex15F2: 5′- GGAGTTCACGGGTGGGA-3′ and Ex15 R2: 5′-GGGTCTCTATGTCGTGGGGG-3′), were designed on the basis of the goat genomic sequence (GenBank accession no. GU440577) using Primer3 software. To amplify PCR products of ∼233 and 117 bp. Primers Ex15F1 and Ex15F2 were 5′ labelled with the fluorescent dye 6-FAM (6-carboxyfluorescein). PCR was carried out with 50 ng of genomic DNA in a total reaction volume of 25 μL containing 10× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 10 pM of forward and reverse primers and 0.5 U of Taq DNA polymerase (Sigma Aldrich). Amplification reactions were performed with an initial denaturation step of 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s X°C (X was 59 for region A and 62.2 for region B) and 25 s at 72°C with a final extension step of 5 min at 72°C. PCR products were screened by 2% agarose gel electrophoresis stained with ethidium bromide and visualized in a gel documentation system (Biorad, USA).

The genotyping of microsatellite markers in the regions A and B was performed by an automated fragment analysis

technique (Scigenom, Ernakulam, India). The fluorescent 5′ end-labelled PCR products (with fluorescent dye, 6-FAM) were run on 3730 XL ABI PRISM automated genetic analyzer (Applied Biosystems, Darmstadt, Germany) and analysed. Microsatellite fragment sizing was performed by the Gene Mapper software ver. 4.0. Allele calling was performed with the software and were checked manually to avoid any false calling of alleles. Sequencing of representative samples from each pattern, obtained by genotype analysis, confirmed that the only nucleotide differences among the PCR products were in the number of GT repeats.

Detection of MAP-specific antibodies by ELISA

Blood samples were collected by jugular puncture; following centrifugation (2500 rpm for 10 min), the sera were separated and stored at −20°C until the use. Serum samples ($n = 150$) were screened for detection of MAP specific antibodies by ELISA kit (ID vet innovative diagnostics, Grabels, France). Optical density (OD) values were measured at 450 nm. Positive and negative sera were included as controls. As per manufacturer's instruction, serum samples with corrected sample / positive control ratio above 60% were considered as positive for paratuberculosis.

Detection of MAP by IS900 PCR

Faecal samples were collected by rectal pinch method. DNA was isolated from faecal sample as per Braunstein *et al.* (2002). IS900 PCR was performed as per the protocols of Halldorsdottir *et al.* (2002). The primer pair used was 5′-GGCCGTCGCTTAGGCTTCGA-3′ and 5′-CGTCGTTAATAACCATGCAG-3′ to amplify a 279-bp PCR product. The PCR mixture (50 μL total volume) comprised 5 μL of DNA, 10× PCR Buffer, 10 pM primers, 1.5 mM for $MgCl_2$, 0.2 mM dNTPs and 0.5 U of *Taq* DNA polymerase. The cycling protocol was an initial denaturation at 94°C for 3 min followed by 35 cycles of 1 min denaturation at 94°C, 25 s primer annealing at 55°C, and extension at 72°C for 1 min. The *IS900* PCR products were separated by electrophoresis in 2% agarose gel, stained with ethidium bromide and visualized by Gel documentation system (Biorad, USA).

Statistical analysis

The allelic and genotype frequencies of A and B microsatellite loci at 3′UTR of *SLC11A1* gene in each genetic group were calculated by direct counting method. Association of the different genotypes in A and B microsatellite regions at the 3′UTR of *SLC11A1* gene with the presence of MAP-specific antibodies in the serum and MAP DNA in the faeces of goats were assessed by chi-square test and the Fisher's exact test by SPSS ver. 21.

Results and discussion

The number of GT repeats found in region A ranged from 10 to 19, eight alleles being identified (A_{10}-GT10, A_{12}-GT12, A_{14}-GT14, A_{15}-GT15, A_{16}-GT16, A_{17}-GT17, A_{18}-GT18 and A_{19}-GT19) with size range of 221–239 bp and 12 genotypes were observed. The 233 bp allele was the most abundant in goat population (0.660). Only two alleles (B_7-GT7 and B_8-GT8) with three genotypes were present in region B. The direct count heterozygosity, unbiased heterozygosity and PIC value for microsatellite A region of *SLC11A1* gene were 0.6833, 0.6995 and 0.5474 and for microsatellite B region were 0.4985, 0.5036 and 0.2485 in goats, respectively (table 1). Liandris *et al.* (2009) detected two microsatellite regions with different GT (dinucleotide) repeat numbers and different sequence motifs in native Greek goats named region A and region B at the 3′UTR of *SLC11A1* gene and detected four alleles (GT14, GT15, GT16 or GT18) in region A and two alleles in region B (GT7 and GT8). In addition to this, four other alleles (GT11, GT12, GT17 and GT19) at region A were recognized in Sarda goats by Piras *et al.* (2011). Korou *et al.* (2010) detected six alleles (GT13, GT14, GT15, GT16, GT17 and GT18) in microsatellite region A at the 3′UTR of *SLC11A1* gene in Greece goats, and two alleles at region B (B_7 and B_8). The allele frequency in region B of the 3′UTR of the *SLC11A1* gene was slightly different to that observed by Korou *et al.* (2010) (53% allele B_7 and 47% allele B_8 in this study versus 45 and 55%; table 1); but, the percentage of goats with B_7/B_7 genotype found among animals evaluated by us was similar to those reported in other goat breeds (17% in this study versus 26% and 16%) (Korou *et al.* 2010; Iacoboni *et al.* 2014). The percentage of goats with B_7/B_8 and B_8/B_8 genotypes, which was found to be in risk of paratuberculosis incidence was higher than those reported in other goat breeds (83% in this study versus 75% reported by Korou *et al.* 2010).

The incidence of paratuberculosis in Malabari, Attapady black and crossbred goat were tested by ELISA and IS900 PCR. The prevalence of paratuberculosis was between 12 and 34%. Lowest incidence was noticed in the native breeds (Attappady Black and Malabari) and highest in crossbreds. Genotype-wise results of MAP infection

Table 1. Direct count heterozygosity, unbiased heterozygosity and PIC value of microsatellite A and B regions at the 3′UTR of *SLC11A1* gene in Indian goats.

Microsatellite region	Region A	Region B
No. of alleles	8	2
Direct count heterozygosity	0.6833	0.4985
Unbiased heterozygosity	0.6995	0.5036
PIC	0.5474	0.2485

Table 2. Genotypes in region A at the 3′UTR of *SLC11A1* gene and paratuberculosis incidence in goats (based on genetic group).

No.	Genotypes	GT repeats	No. of animals	ELISA positive	IS900 PCR positive
Malabari goats					
1	233/233	GT16/GT16	22	3	9
2	221/233	GT10/GT16	11	1	1
3	233/237	GT16/GT18	8	0	1
5	231/233	GT15/GT16	3	0	1
6	233/235	GT16/GT17	2	1	1
7	233/239	GT16/GT19	3	0	0
8	221/221	GT10/GT10	1	1	1
Attapady black goats					
1	233/233	GT16/GT16	16	1	1
2	221/233	GT10/GT16	5	0	0
3	233/237	GT16/GT18	4	1	1
4	231/235	GT15/GT17	8	2	2
5	231/233	GT15/GT16	1	0	0
6	233/235	GT16/GT17	2	0	0
7	221/237	GT10/GT18	5	1	1
8	225/237	GT12/GT18	2	0	0
9	233/239	GT16/GT19	1	0	0
10	229/231	GT14/GT15	2	1	1
11	225/225	GT12/GT12	1	0	0
Crossbred goats					
1	233/233	GT16/GT16	32	5	11
2	221/233	GT10/GT16	7	1	2
3	233/237	GT16/GT18	9	0	2
4	231/233	GT15/GT16	1	0	0
5	225/237	GT12/GT18	3	1	2
6	233/239	GT16/GT19	1	0	0

Table 3. Genotypes in region B at the 3′UTR of *SLC11A1* gene and paratuberculosis incidence in goats (based on genetic group).

	Genotypes	GT repeats	No. of animals	ELISA positive	IS900 PCR positive
Malabari goats					
1	117/117	GT7/GT7	12	0	2
2	117/119	GT7/GT8	26	3	5
3	119/119	GT8/GT8	12	2	8
Attapady black goats					
1	117/117	GT7/GT7	6	0	0
2	117/119	GT7/GT8	38	1	2
3	119/119	GT8/GT8	3	4	4
Crossbred goats					
1	117/117	GT7/GT7	8	0	0
2	117/119	GT7/GT8	42	11	14
3	119/119	GT8/GT8	3	2	4

are presented in tables 2 and 3. The association analysis between diagnostic tests results and polymorphisms in regions A and B of the 3′UTR of the caprine *SLC11A1* gene indicated that genotypes of A region had no significant effects on paratuberculosis resistance/incidence in goats, whereas region B showed a significant association ($P < 0.01$) with paratuberculosis incidence (table 4). Of the three different genotypes in the B region (117/117 (B_7B_7), 117/119 (B_7B_8) and 119/119 (B_8B_8)), those genotypes with 119 bp (B_8) alleles (both in homozygous and heterozygous conditions) showed significant association with paratuberculosis incidence by both diagnostic methods ($P < 0.01$). The details of the association between different genotypic variants of region B and paratuberculosis incidence in goats are given in table 5. Korou *et al.* (2010) reported that the presence of B_7 allele was significantly associated with absence of MAP specific antibodies in goats, but they did not find association between absence/presence of MAP antibodies with polymorphisms in region A, as in the present study. Similar associations of *SLC11A1* gene polymorphisms with susceptibility of humans and bovines to *Mycobacterium* spp. and *Brucella*

Table 4. Association of the diagnostic test results with the genotypes of the region A and region B at the 3′UTR of *SLC11A1* gene.

	Microsatellite region	Test	*P*
1	Region A	ELISA	0.836^{NS}
2	Region A	IS900 PCR	0.508^{NS}
3	Region B	ELISA	<0.01*
4	Region B	IS900 PCR	<0.01*

Statistical significance was considered for $P < 0.01$ based on chi-square test. Level of significance $P < 0.01$. *Significant at $P < 0.01$; NS, non significant

Table 5. Association of the results for ELISA and IS900 PCR with the presence of the B8 allele in homozygous and heterozygous conditions, based on the chi-square test.

	Genotype	ELISA		IS900 PCR	
		+	–	+	–
1	B_7/B_7	0	26	2	24
2	B_7/B_8 and B_8/B_8	23	101	37	87
		$P < 0.01$		$P < 0.01$	

spp. were reported (Bellamy 1998; Barthel *et al.* 2001; Capparelli *et al.* 2007). The homozygous B_7/B_7genotype was reported to be associated with increased expression of the *SLC11A1* and *IL-1α* genes indicating increased *in vitro* responsiveness and therefore, resistance of mononuclear-derived macrophages to MAP infection (Taka *et al.* 2013).

The results of the present study will supplement the information available for the role of *SLC11A1* gene in disease resistance/susceptibility and will be useful in further studies to determine the markers for selection of paratuberculosis-resistant animals. Further investigations are necessary to unravel regulation of *SLC11A1* gene expression based on the genetic variants after intracellular pathogen infection in native goats. Thus, the physiological and biochemical functions, together with the results obtained in the current research, indicate that the *SLC11A1* gene might play a crucial role in disease resistance in goats. The results obtained in the research open a promising opportunity to use these markers as one of the tool in a selective breeding programme to control paratuberculosis in goats.

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