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DNA Polymorphism in SLC11A1 Gene and its Association with Brucellosis Resistance in Indian Zebu (*Bos indicus*) and Crossbred (*Bos indicus*×*Bos taurus*) Cattle

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ABSTRACT : The PCR- restriction fragment length polymorphism (RFLP) in and around TM4 of SLC11A1 gene and its association with the incidences of brucellosis in Hariana breed (*Bos indicus*) and Holstein Friesian crossbred (*Bos indicus*×*Bos taurus*) cattle was examined. A fragment of 954 bp encoding the TM4 was amplified, and RFLP was identified by digestion of the amplicon independently with *Alu*I and *Taq*I. The amplicon (GenBank Acc. No. AY338470 and AY338471) comprised of a part of exon V (<59 bp) and VII (62>), and entire intron 5 (423 bp), exon VI (71 bp) and intron 6 (339 bp). Digestion with *Alu*I revealed the presence of two alleles viz, A (281, 255, 79 and 51 bp) and B (541, 255, 79 and 51 bp). The frequency of A allele was estimated as 0.80 and 0.73 in Hariana and crossbred cattle, respectively. Due to presence of a polymorphic *Taq*I site at intron 5, two alleles: T (552 and 402 bp) and Q (231, 321 and 402 bp) were identified. The frequency of T allele was estimated as 0.96 and 0.97, respectively. For association study, on the basis of serological tests and history of abortion, the animals were grouped into "affected" and "non-affected". However, no association could be established with the observed RFLPs. (**Key Words :** Genetic Resistance, DNA Polymorphism, SLC11A1, Zebu Cattle, Crossbred Cattle, Brucellosis)

INTRODUCTION

Solute linked carrier 11A1 (SLC11A1), a transmembrane protein (also referred as Natural Resistance Associated Macrophage Protein 1, NRAMP1), is one of the potential candidate genes that confer innate resistance against various intracellular pathogens. The role of SLC11A1 was first demonstrated by the fact that mice carrying a point mutation G169D (Vidal et al., 1996), located within the fourth transmembrane domain (TM4), were susceptible towards a number of antigenically different intracellular pathogens namely *Salmonella typhimurium* (Plant and Glynn, 1976), *Leishmania donovani* (Bradley et al., 1979) and *Mycobacterium tuberculosis* (Gros et al., 1981). The G169D point mutation, observed in mice, could not be observed in any mammalian species. Several studies, however, have been initiated to identify new DNA polymorphisms and to ascertain its association with the host resistance/susceptibility against different intracellular pathogens in various species including human (Liu et al., 1995; Abel et al., 1998; Bellamy et al., 1998; Jin et al., 2009), cattle (Adams and Templeton, 1998; Horin et al., 1999; Kumar et al., 1999; Martinez et al., 2008), buffalo (Ganguly et al., 2008), sheep (Matthews and Crawford, 1998), pig (Zhang et al., 2000) and horse (Horin and Matiasovic, 2000).

Brucellosis is one of the major zoonotic infections worldwide. The causative organism, *Brucella abortus* is a

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facultative intracellular bacteria that is capable of surviving and replicating within the phagosome. Macrophages isolated from naturally susceptible and resistant animals exhibited a differential ability in controlling the intracellular replication of Brucella organisms (Crocker et al., 1984; Price et al., 1990; Campbell and Adams, 1992). In cattle, a $(GT)_{13}$ microsatellite allele at 3'UTR of SLC11A1 has a significant association with the natural resistance to brucellosis (Adams and Templeton, 1998). In an *in vitro* macrophage challenge study, Barthel et al. (2001) showed that $(GT)_{13}$ allele restricted the intracellular replication of Brucella organisms. On the contrary, our group (Kumar et al., 2005) and more recently Paixao et al. (2007) demonstrated a lack of association of 3′ UTR polymorphisms with the resistance against bovine brucellosis. On the other hand, Capparelli et al. (2007a, 2007b) reported a significant association of polymorphisms at 3′ UTR of SLC11A1 gene with resistance/susceptibility to brucellosis in buffalo. Nevertheless, polymorphisms in other regions of SLC11A1 gene can serve as a potential marker (Liu et al., 1995; Abel et al., 1998; Bellamy et al., 1998). Further, TM4 is known to play an important role in positioning the NRAMP1 protein within the phagosomal membrane (Malo et al., 1994). The present study was undertaken to identify polymorphism in and around TM4, and to investigate its association with the incidences of brucellosis in Indian zebu (*Bos indicus*) and Holstein Friesian crossbred (*Bos indicus* × *Bos taurus*) cattle.

MATERIALS AND METHODS

Animals

Animals belonging to Hariana breed (*Bos indicus*) and Holstein Friesian crossbred were included in the study. The crossbred cattle comprised of various genetic constitutions i.e., (1/2 F X 1/2 H); (1/2 F X 1/4 B X 1/4 H); (1/2 F X1/4 J X 1/4 H), where F stands for Holstein Friesian, B for Brown Swiss, J for Jersey and H for Hariana. A random sample of 50 animals from each breed was used for the estimation of gene and genotype frequencies. In addition to the animals mentioned above, 20 animals from Hariana breed and 30 from crossbred cattle having additional records pertaining to date of birth, parity, calving, abortion and vaccination were included for association study.

Isolation of genomic DNA

Genomic DNA was isolated from the venous blood using standard phenol chloroform extraction method (Sambrook et al., 1989).

PCR amplification of TM4 of SLC11A1 gene

A pair of primer (NRE5F 5′ TCCGACATGCAGG AAGTCAT 3′; NRE7R 5′ GCCGAAGGTCAAGGCCAT TATGG 3′) were designed on the basis of aligned sequences of SLC11A1 gene of cattle cDNA (GenBank Acc. No. U12862), human cDNA (GenBank Acc. No. D50403) and human genomic sequences (GenBank Acc. No. AF229163) to amplify a fragment comprising intron 5, exon VI, intron 6 and a part of exon V and VII, corresponding to TM4. The PCR reaction was carried out in 50 µl mixture containing 2.0 mM of $MgCl_2$, 1.0 U of *Taq* DNA polymerase, 200 µM of dNTPs, 5 pM of each primer and approximately 100 ng of genomic DNA as template. The PCR product was checked by agarose gel (1%) electrophoresis in 1X TAE buffer after staining with ethidium bromide.

Nucleotide sequencing and sequence analysis

In order to confirm the restriction patterns, the amplicons from two random representative animals of Hariana breed of cattle were sequenced using automated DNA sequencer (ABI Prism Ver 2.0) following Sanger's dideoxy chain termination method. The sequences obtained were subjected to BLAST analysis (www.ncbi.nlm.nih.gov/BLAST). Using MAPDRAW program of Lasergene Software (DNASTAR), the presence of potential recognition sites for restriction enzyme (RE) was analyzed. The nucleotide and deduced amino acid sequences were compared with that of other species available in the GenBank using the Clustal method of MEGALIGN programme of DNASTAR.

PCR-Restriction fragment length polymorphism (PCR-RFLP)

In order to detect the PCR-RFLP, the amplicon was digested independently with 10 units of *Alu*I and *Taq*I. The digestion was carried out overnight in a water bath at 37°C and 65°C for *Alu*I and *Taq*I, respectively. For restriction fragment analysis, digested product was run on 2.5% and 1.5% agarose gel, respectively in 1X TBE buffer for 2-3 h at 5 V/cm.

Association study

For association study, information pertaining to date of birth, parity, calving, abortion and vaccination was collected. To avoid the interference of brucella antibodies due to vaccination, only non-vaccinated animals were chosen (20 animals of Hariana breed and 30 of crossbred cattle). These animals were then screened using three different serological tests viz., Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Test (STAT) and Indirect Enzyme Linked Immunosorbent Assay (Indirect ELISA) as per standard method described by Kumar et al. (2005). On the basis of results from serological tests and history of abortion, the animals were divided into two groups viz., affected and non-affected. The animals that were serologically positive in all the three tests and had a history of abortion were

Table 1. Size differences of intron 5 and 6 in different mammalian species

Species	Intron 5	Intron 6	GenBank Acc No. and Ref.
Cattle (*Bos indicus*)	423	339	AY338470, AY338471
Cattle (*Bos taurus*)	423	340	AY398762, AY398763 (Coussens et al., 2004)
Buffalo	423	339	AY860618, AY 707989, AY860620 (Ganguly et al., 2011 in press)
Human	418	334	AF229163 (Marquet et al., 2000)
Pig	367	333	AY368472, AY368473 (Wu et al., 2007)
Dog	425	336	AF091049 (Altet et al., 2002)

grouped as "affected". Whereas, animals that were serologically negative in all the three tests and had no history of abortion till third lactation or completion of the study, which ever was later, were grouped as "non-affected".

RESULTS

Amplification and characterizations of TM4

Agarose gel electrophoresis of the amplicon as expected revealed an amplification of a fragment approximately 954 bp. The size of the amplicon was further confirmed by nucleotide sequencing (GenBank Acc. No. AY338470 and AY338471). As predicted, the amplicon comprised a part of exon V (<59 bp) and VII (62>), and the entire intron 5 (423 bp), exon VI (71 bp) and intron 6 (339 bp). A comparison of the obtained nucleotide sequences with the corresponding region of the available genomic sequences of different species (viz. taurine cattle, human, pig and dog), revealed a variation in the size of the fragment of 954, 944, 892 and 953 bp, respectively. The size of the corresponding exonic region of 192 bp was conserved across the species and the variation in size of the amplicons was mainly due to difference in the size of the intron (Table 1). While comparing the 192 bp exonic region of Hariana cattle with the corresponding region of *Bos taurus* cattle and buffalo, only one and two synonymous nucleotide substitutions were found, respectively. However, on comparison with other species homologues, a total of 63 nucleotide substitutions were observed, amongst them 12 were non-synonymous.

The phylogenetic tree (Figure 1) based on the deduced amino acid sequence showed that *Bos indicus* (Hariana) cattle are closely related to *Bos taurus* cattle and with buffalo form a single clad. However, sheep are interestingly placed in a separate group with chicken, and pigs formed a distinct clad from the remaining species.

PCR-RFLP

Restriction fragment length polymorphisms (RFLP) were identified by restriction digestion of the amplicon independently with *Alu*I and *Taq*I.

AluI/PCR-RFLP

Restriction digestion and sequence analysis indicated the presence of five *Alu*I sites in the amplicon of which the RE site at intron 6 was polymorphic (Figure 2a). *Alu*I digestion should generate six restriction fragments (viz. 281, 260, 255, 79, 51 and 28 bp). However, due to inherent limitation of agarose gel electrophoresis the fragment of 28 bp could not be resolved and the fragments of 260 and 255 bp co-migrated as a single band. Consequently, two alleles were distinguishable viz, A (281, 255, 79 and 51 bp) and B (541, 255, 79 and 51 bp). A total of 35 and 31 AA, 10 and 11 AB, and 5 and 8 BB animals were identified in Hariana and crossbred cattle. The frequencies of A allele were estimated as 0.80 and 0.73, respectively (Table 2).

TaqI/PCR-RFLP

Digestion of PCR product with *Taq*I revealed two restriction patterns. Further, nucleotide sequence analysis of the amplicon demonstrated the presence of two *Taq*I sites in which the RE site present at intron 5 was polymorphic (Figure 2b). Accordingly, two alleles were identified: T

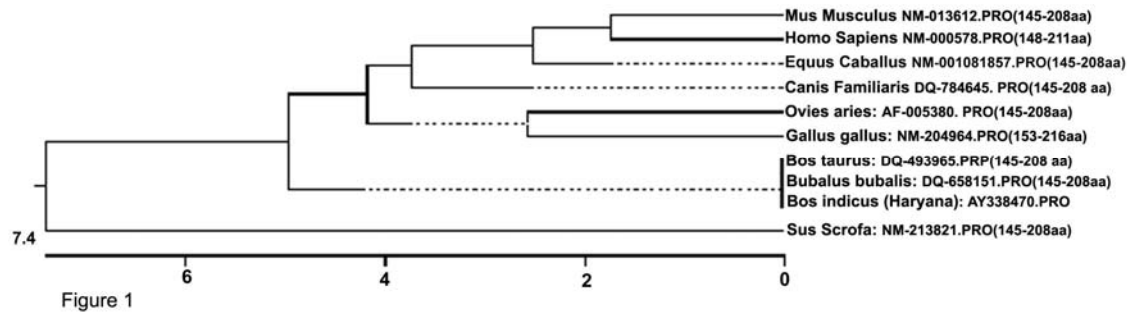

Figure 1. Phylogram based on the deduced amino acid sequences from exon V-VII of SLC11A1 gene.

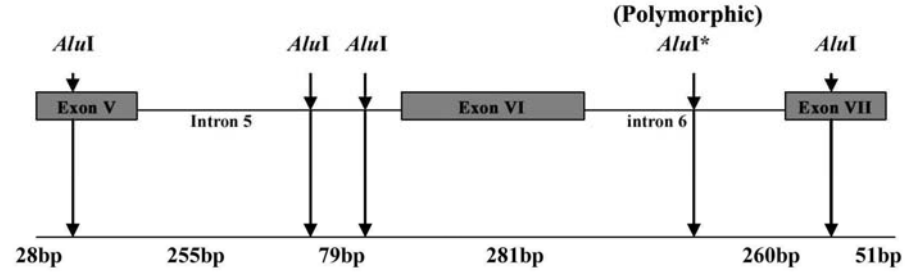

Figure 2a

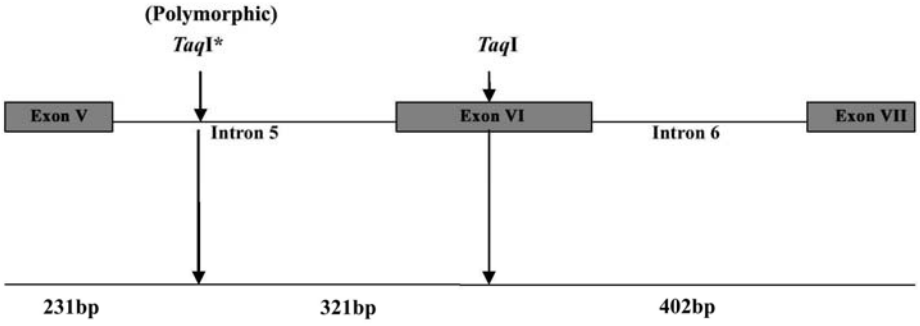

Figure 2b

Figure 2. Schematic representation of restriction enzyme analysis of amplicon encompassing exon V-VII of SLC11A1 gene with *Alu* I (a) and *Taq* I (b).

(552 and 402) and Q (231, 321 and 402). Among Hariana zebu and crossbred cattle, 46 and 47 TT and 4 and 3 TQ animals were found, respectively. However, none of the animals were homozygous QQ. The allelic frequency of T was estimated as 0.96 and 0.97, respectively (Table 3).

Association study

On the basis of serological tests and abortion history, a total of nine and seven crossbred cattle were grouped as "affected" and "non-affected". In Hariana cattle, none of the animals could be grouped as "affected" and only five of them could be grouped as "non-affected". In *Taq*I PCR-RFLP, all the animals belonging to both affected and non-affected group were of homozygous TT genotype. However, in case of *Alu*I PCR-RFLP all animals except two were homozygous AA genotype. Accordingly, no association could be established between polymorphism in SLC11A1 gene and the incidences of brucellosis.

DISCUSSION

This is the first study of its kind trying to associate PCR-RFLP (exon V-VII) polymorphism with the incidences of brucellosis in cattle. One polymorphic restriction site for *Alu*I and *Taq*I has been identified in intron 6 and 5. In both the breeds, homozygous AA genotype was more frequent than AB and BB. In case of *Taq*I, homozygous QQ genotype was not observed in either breed. However, PCR-RFLP results showed no variation within the breed in any coding region.

In the present study, we could not find any association between the observed PCR-RFLP and the incidence of brucellosis. It could be argued that in grouping animals for the association study we might have included false positive and/or false negative animals. However, three serological tests and history of abortion were used to exclude false positive and false negative. Nonetheless, it is known that

Table 2. Gene and genotype frequencies of *Alu*-I PCR-RFLP

Sl. No.	Breeds	Total no. of individuals	Genotype frequency			Gene frequency	
			AA	AB	BB	A	B
1.	Crossbred	50	0.62 (n = 31)	0.22 (n = 11)	0.16 (n = 8)	0.73±0.04	0.27±0.04
2.	Hariana	50	0.70 (n = 35)	0.20 (n = 10)	0.10 (n = 5)	0.80±0.04	0.20±0.04

Table 3. Gene and genotype frequencies of *Taq*-I PCR-RFLP

Sl. No.	Breeds	Total no. of individuals	Genotype frequency			Gene frequency	
			TT	TQ	QQ	T	Q
1.	Crossbred	50	0.94 (n = 47)	0.06 (n = 3)	0.00 (n = 0)	0.97±0.017	0.03±0.017
2.	Hariana	50	0.92 (n = 46)	0.08 (n = 4)	0.00 (n = 0)	0.96±0.0195	0.04±0.0195

none of the serological tests used for detection of brucellosis are full proof. For example, indirect-ELISA in which Brucella LPS was used as an antigen suffers from two limitations. Firstly, this test sometimes could not distinguish between the animals infected and vaccinated with *B. abortus* strain19 (Nielsen et al., 1992) but in the present study only non-vaccinated animals were included. Secondly, smooth LPS of several bacteria such as *Yersinia enterocolitica* (Ahvonen et al., 1969), *Vibrio cholerae* (Sandulache and Marx, 1978) and Salmonella serotypes Kauffmann white group N (Corbell, 1975) are also reported to cross-react with *B. abortus*. Consequently, the available history of dams, having late gestational abortion (5-8 months) typical to brucellosis was used to exclude such false positive due to cross-reacting bacteria. Further, we had confirmed the presence of brucella organism by culture examination (in crossbred cattle only) of heart blood, spleen, lung, stomach of fetuses (data not shown).

In mice SLC11A1 was not an important determinant in conferring natural resistance against brucellosis as compared to that against Salmonella, Leishmania, and Mycobacterium (Guilloteau et al., 2003). We (Kumar et al., 2005) and others (Paixao et al., 2007) have also demonstrated the lack of association of $(GT)_n$ 3′ UTR microsatellite polymorphisms with the resistance against bovine brucellosis either in experimental or natural infection. Nevertheless, these findings can not exclude the possible role of bovine SLC11A1 gene restricting intracellular replication of *Brucella abortus*. As on the contrary, several other studies (Borriello et al., 2006; Capparelli et al., 2007a; Capparelli et al., 2007b) revealed a strong association of $(GT)_n$ microsatellite polymorphisms with the resistance/susceptibility against brucellosis in water buffalo. Recently, we have also demonstrated that macrophages from $(GT)_{13}$ buffaloes produced more nitric oxide and H_2O_2 when challenged with brucella LPS (Ganguly et al., 2008). It is plausible that more than a single gene is responsible for determining the resistance/ susceptibility to brucellosis. The classical breeding studies conducted by Templeton et al. (1988) showed that two or more genes are involved in controlling Brucella infection. A M17T mutation in bison prion protein in homozygous condition was found to be associated with the seropositivity to Brucella species (Seabury et al., 2005). SLC11A1 may also have pleiotropic effect on other genes. For instance, SLC11A1 is known to involve in the high level of expression of MHC and surface antigen expression in mice (Denis et al., 1988; Lang et al., 1997).

In conclusion, we identified *Alu*I and *Taq*I polymorphisms in SLC11A1 gene in cattle, but association of the observed allelic variants with the resistance/ susceptibility to brucellosis could not be established. Future studies need to be directed to explore polymorphisms throughout the entire SLC11A1 gene and to ascertain their suitability as potential genetic marker for brucellosis resistance.

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