

CHARACTERISATION OF EXON 9 OF SOLUTE CARRIER FAMILY 11 MEMBER A1 GENE IN VECHUR CATTLE

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Abstract: The solute carrier family 11 member A1 (*SLC11A1*) gene has been associated with natural resistance to intracellular pathogens such as Brucella, Salmonella, Leishmania and Mycobacterium in several species including bovine and plays a critical role in elimination of pathogens by generating hydroxyl free radicals. The objective of the present study was to investigate the polymorphism in exon 9 of *SLC11A1* gene in Vechur cattle, one of the dwarf cattle of India which is known for its disease resistance. A 198 bp fragment containing exon 9 of the gene was amplified by polymerase chain reaction (PCR). The amplicons upon single strand conformation polymorphism (SSCP) analysis revealed two different banding patterns. A novel non synonymous SNP (g.46C>T) with predominance of CC genotype was also detected in Vechur cattle. These results suggest that there exists a considerable genetic variation at *SLC11A1* locus and further association studies may help in development of a PCR based genotyping assay to select cattle with better immunity to intracellular pathogens.

Key words: *SLC11A1*, Vechur, Disease resistance, Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP)

Introduction

Dairy industry is affected by various infectious diseases such as Brucellosis, Paratuberculosis, Salmonellosis caused by various intracellular pathogens. These infections has a significant economic impact and causes substantial financial losses every year. Due to a more efficient host immunological defence, native breeds are found to be resistant against various pathogens. These resistance may be associated with one or more host genes. Thus, it is increasingly

important to study those natural resistance against disease in different bovine breeds as it contributes to the control and eradication of diseases through genetic selection.

One of the candidate genes studied in livestock is *SLC11A1* gene. *SLC11A1* gene formerly known as Natural Resistance Associated Macrophage Protein 1 (*NRAMP1*) gene encodes a protein with 12 transmembrane domains which is involved in the transport of divalent cation such as Fe^{2+} , Mn^{2+} , Zn^{2+} , Mg^{2+} and Co^{2+} ions (Vidal et al., 1996; Goswami et al., 2001). The *SLC11A1* gene delivers increased concentration of iron from cytosol into the phagolysosome which catalyses Fenton and Haber-Weiss reaction generating oxygen intermediates favouring bacterial killing (Goswami et al., 2001). *SLC11A1* has many pleiotropic effects on macrophage function that includes enhanced tumor necrosis factor- α , interleukin- 1β , inducible nitric oxide synthase and MHC class II expression which are important in resistance to intra cellular pathogens such as *Mycobacterium tuberculosis* (Awomoyi, 2007).

Various studies suggested variations in both coding and noncoding sequences of *SLC11A1* gene in many species. Mutations in the coding regions result in alteration of *SLC11A1* function leading to variation in disease resistance by increasing or decreasing the rate of transcription. The polymorphisms in the promoter could lead to altered expression of the gene (Nicholas, 2012). A mutation at position 169 of the protein (glycine to aspartic acid) had resulted in loss of function of *SLC11A1* in mice (Vidal et al., 1993). Microsatellite polymorphisms detected in the 3'UTR of the *SLC11A1* gene in ruminants were found to be associated with resistance to *Brucella abortus*, *Mycobacterium bovis* and *Mycobacterium avium subsp. paratuberculosis* (Barthel et al., 2001; Borriello et al., 2006; Capparelli et al., 2007a; Kadarmideen et al., 2011; Korou et al., 2010; Martinez et al., 2008, 2010; Pinedo et al., 2009; Reddacliff et al., 2005; Taka et al., 2013; Taka et al., 2015).

According to the National Bureau of Animal Genetic Resources (NBAGR), out of 39 recognised cattle breeds in India, Vechur cattle is the only recognised cattle breed from Kerala. The Vechur Cattle was named after the village Vechur in Kottayam district of the state of Kerala in India, where it was supposed to have evolved. The extremely small size, low feed requirements and high disease resistance make these cattle reliable. Vechur cows were observed to have low incidence of diseases and are less prone to mastitis, parasites, or foot and mouth disease (Iype and Venkatachalapathy, 2001).

Since Vechur cattle, the indigenous cattle of Kerala, are well known for their disease resistance, the objective of the present study was to characterize the exon 9 of *SLC11A1* gene in Vechur cattle.

Materials and Methods

Sample collection and isolation of genomic DNA

Blood samples were collected from the animals maintained at the Vechur Cattle Conservation Centre, Mannuthy. Five millilitres each of blood samples were collected in vacutainers coated with EDTA from the jugular vein of 60 Vechur adult cattle. DNA was isolated by the standard phenol chloroform extraction method (Sambrook and Russell, 2001) with minor modifications.

PCR amplification of exon 9 of SLC11A1 gene

A pair of primers were (EX9F 5'TCCTTTTACCTTCGTAGTCTCG 3' and EX9R 5' GAATATATGGGGTGTGCCTCA 3') were designed using the bioinformatics tool Primer 3 (Untergasser *et al.*, 2012) based on the reference sequence (GenBank Acc. No. DQ493966.1). A region corresponding to 198 bp of exon 9 of *SLC11A1* gene was amplified by PCR. The PCR reaction was carried out in 25 µl mixture containing 1.5 mM of MgCl₂, 0.5 U of Taq DNA Polymerase, 200 µM of dNTP, 10 pM of each forward and reverse primer and 50 ng of genomic DNA as template. The thermal cycling profile for the reaction includes initial denaturation for 3 min at 95°C, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55 °C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 5min. The amplified products were then resolved in 2% agarose gel electrophoresis and visualised in UV transilluminator (Bio Rad, USA) after staining with ethidium bromide.

Single strand conformation polymorphism analysis

SSCP was conducted by mixing 10 µl of PCR product with 15 µl of denaturing buffer (9.5 ml formamide, 0.4 ml of 0.5M EDTA, 2.5 mg xylene-cyanole and 2.5 mg bromophenol blue). The mixture was then incubated at 95°C for 10 min and immediately chilled on ice. Denatured PCR products were separated by 12% polyacrylamide gel electrophoresis (acrylamide:bisacrylamide = 29:1). The gel was run at 130 voltage for 17 hrs time at 4 °C, in a vertical electrophoresis apparatus (Hoefer, USA). The SSCP patterns were visualised using silver nitrate staining, photographed and analysed.

Nucleotide sequencing of SSCP alleles

Representative PCR products showing different banding patterns in SSCP were sequenced using respective forward and reverse primer to detect variations in nucleotides. Sequencing was performed by automated sequencer using Sanger's dideoxy chain termination method at Sci Genom Labs Pvt. Ltd., Cochin, India, and aligned with other sequences in GenBank employing BLASTn. For comparative analysis, the *SLC11A1* gene reference sequences from 8 mammalian species were retrieved from the GenBank database. Multiple Sequence Alignment was performed using the bioinformatics tool Clustal Omega. The *SLC11A1* gene sequences of different species were analysed using the 'MegAlign' tool of

Lasergene Software (DNASTAR, Madison, WI, USA) to generate phylogenetic tree.

Statistical analysis

The genotypes were identified by observing the SSCP patterns of each sample in the gels which was further confirmed by sequencing. The genotype frequency and allele frequency were calculated using the formulae:

$$\text{Genotype frequency} = \frac{\text{No of individuals with 'particular genotype'}}{\text{Total no of individuals in the population}}$$

$$\text{Allele frequency} = \frac{\text{No of copies of a given allele}}{\text{Sum of counts of all alleles in the population}}$$

Results and Discussion

Single strand conformation polymorphism analysis

In order to ascertain the polymorphism in 198 bp fragment (comprising of partial intronic region (8 and 9) and complete exon 9), the PCR product was subjected to SSCP-PAGE. Silver staining revealed two unique SSCP banding patterns. Pattern 1 consisted of two bands and pattern 2 consisted of three bands which were identified as CC and CT respectively (Figure1a). Sequencing results confirmed the presence of a novel SNP with C to T transition in the exon 9 of *SLC11A1* gene (Figure1b). In *SLC11A1* gene, there are reports of coding region mutations in various exons. *Bagheri et al. (2015)* reported a novel mutation in exon 11 of the *SLC11A1* gene of Holstein dairy cattle and this SNP had a significant effect on the occurrence of clinical mastitis. SNP in exon 4 and intron 4 were reported by *Cheng et al. (2015)*, suggested that exon 4 polymorphism was corresponding to a non synonymous mutation (alanine to threonine), which was associated with tuberculosis in Holstein cattle. *Korou et al. (2010)* identified two polymorphic regions in 3'UTR of the *SLC11A1* gene and their significant association with the detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) antibody by ELISA in goats.

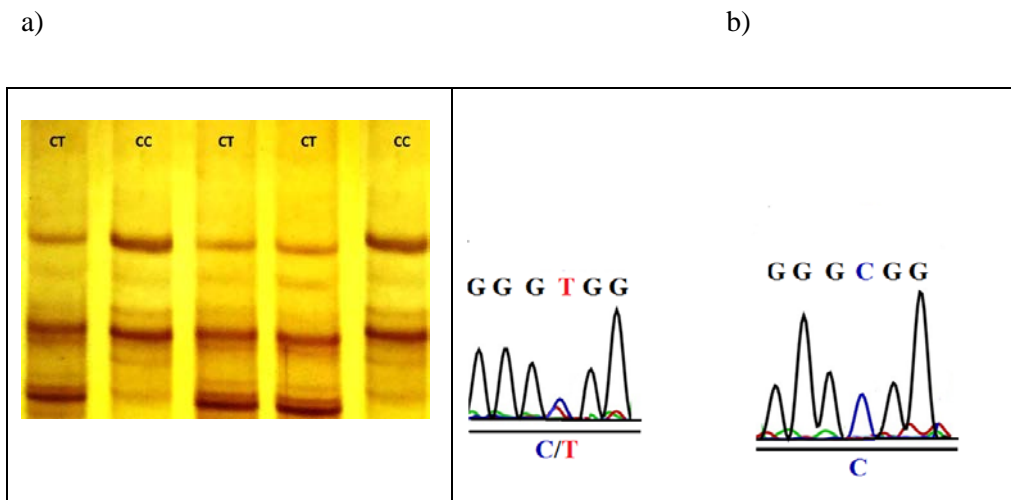


Figure 1. PCR-SSCP analysis of exon 9 of *SLC11A1* gene. (a) SSCP banding pattern of 198 fragment of exon 9 of *SLC11A1* gene (b) Sequence maps showing SNP g.46C>T

Using the ExPASy Translate tool the amino acid sequences (partial protein sequence corresponding to the exon 9) were predicted, for each genotype. The predicted sequence suggested that the novel SNP, g.46C>T causes a non synonymous mutation, with substitution of alanine (GCG) by Valine (GTG).

The frequency of CC genotype was found to be 0.6 and CT was 0.4 in the population studied. The allele frequencies of C and T allele were found to be 0.8 and 0.2 respectively. From these values we can infer that the CC genotype showed high frequency and the C allele was found to be predominant.

Nucleotide sequence analysis

Clustal Omega analysis of the variant pattern of SSCP showed an SNP at 888th position compared to the reference sequence (GenBank Acc. No. NM_174652.2). The sequences were subjected to identity/divergence analysis using MegAlign tool. The 198 bp sequences obtained from C and T alleles of Vechur cattle were compared with the sequences of other mammalian species available in the NCBI database (Figure 2). The designated T allele showed 100 percent identity with the sequence of other cattle whereas 99.4 percent similarity with designated C allele (Figure 3). Most distant species identified was *Sus scrofa* (94.3%).

CLUSTAL OMEGA (1.2.1) multiple sequence alignment

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Sus scrofa          tctcgaagaggtagaccggaccogcogggaggacatccgagaagccaacatgtacttcctg
Cervus elephus     tctcgaagaggtagaccggaccogcogggctgacatccgagaagccaacatgtacttcctg
Bubalus bubalis    tctcgaagaggtagaccggaccogcogggaggacatccgagaagccaacatgtacttcctg
Capra hircus       tctcgaagaggtagaccggaccogcogggaggacatccgagaagccaacatgtacttcctg
Ovis aries         tctcgaagaggtagaccggaccogcogggaggacatccgagaagccaacatgtacttcctg
Bos taurus         tctcgaagaggtagaccggaccogcogggaggacatccgagaagccaacatgtacttcctg
Bos indicus        tctcgaagaggtagaccggaccogcogggaggacatccgagaagccaacatgtacttcctg
Vechur T allele    tctcgaagaggtagaccggaccogcogggaggacatccgagaagccaacatgtacttcctg
Vechur C allele    tctcgaagaggtagaccggaccogcogggaggacatccgagaagccaacatgtacttcctg
***** * *****

Sus scrofa          attgaatccaccatogccctgttctgtctccttctcatcaacctcttgtcatggctgctc
Cervus elephus     attgaagctaccatogccctgtctgtctcttctcctcatcaacctcttgtcatggctgctc
Bubalus bubalis    attgaagccaccatogccctgtctgtctccttctcatcaacctcttgtcatggctgctc
Capra hircus       attgaagccaccatogccctgtctgtctccttctcatcaacctcttgtcatggctgctc
Ovis aries         attgaagccaccatogccctgtctgtctccttctcatcaacctcttgtcatggctgctc
Bos Taurus         attgaagccaccatogccctgtctgtctccttctcatcaacctgttgtcatggctgctc
Bos indicus        attgaagccaccatogccctgtctgtctccttctcatcaacctgttgtcatggctgctc
Vechur T allele    attgaagccaccatogccctgtctgtctccttctcatcaacctgttgtcatggctgctc
Vechur C allele    attgaagccaccatogccctgtctgtctccttctcatcaacctgttgtcatggctgctc
***** * *****

Sus scrofa          ttgggcaagccttctacaagcaaaccaaccaggctgcg
Cervus elephus     ttgggcaagccttctacaagcaaaccaaccaggctgcg
Bubalus bubalis    ttgggcaagccttctacaagcaaaccaaccaggctgcg
Capra hircus       ttgggcaagccttctacaagcaaaccaaccaggctgcg
Ovis aries         ttgggcaagccttctacaagcaaaccaaccaggctgcg
Bos taurus         ttgggcaagccttctacaagcaaaccaaccaggctgcg
Bos indicus        ttgggcaagccttctacaagcaaaccaaccaggctgcg
Vechur T allele    ttgggcaagccttctacaagcaaaccaaccaggctgcg
Vechur C allele    ttgggcaagccttctacaagcaaaccaaccaggctgcg
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Figure 2. Alignment of nucleotide sequence of exon 9 of *SLC11A1* gene of *Bos taurus*, *Bos indicus*, *Capra hircus*, *Ovis aries*, *Cervus elephus*, *Sus scrofa* and *Bubalus bubalis* using Clustal Omega

		Percent Identity										
		1	2	3	4	5	6	7	8	9		
Divergence	1	█	99.4	99.4	98.1	95.6	98.1	98.1	98.7	98.1	1	Bubalus bubalis FJ827149.1.seq
	2	0.6	█	100.0	97.5	96.2	97.5	97.5	98.1	97.5	2	Capra hircus NM_001285694.1.seq
	3	0.6	0.0	█	97.5	96.2	97.5	97.5	98.1	97.5	3	Ovis aries AF005380.1.seq
	4	1.9	2.6	2.6	█	93.7	96.2	96.2	96.9	96.2	4	Cervus elaphus AF005379.1.seq
	5	4.5	3.9	3.9	6.6	█	94.3	94.3	94.3	94.3	5	Sus scrofa NM_213821.2.seq
	6	1.9	2.6	2.6	3.9	5.9	█	100.0	99.4	100.0	6	Bos Taurus DQ493965.1.seq
	7	1.9	2.6	2.6	3.9	5.9	0.0	█	99.4	100.0	7	Bos indicus DQ493966.1.seq
	8	1.3	1.9	1.9	3.2	5.9	0.6	0.6	█	99.4	8	Exon 9 C allele vechur cattle.seq
	9	1.9	2.6	2.6	3.9	5.9	0.0	0.0	0.6	█	9	Exon 9 T allele vechur cattle.seq
		1	2	3	4	5	6	7	8	9		

Figure 3. SLC11A1 nucleotide percent identity and divergence in eight species

Phylogenetic analysis

The phylogenetic tree from 198 bp fragment revealed close evolutionary relationship between designated C and T alleles and other cattle as they formed a common cluster (Figure 4). This was expected as they had a high percent similarity in their sequences. The phylogenetic tree showed two major branches at the primary node for pig and ruminants. Three unique branches were formed for cattle, buffalo and goat.

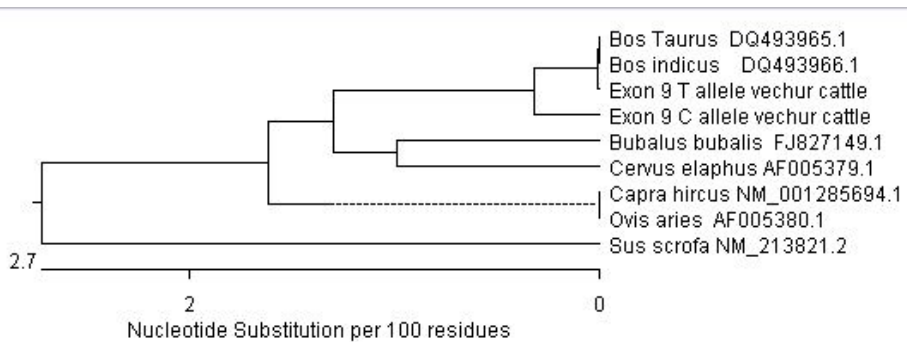


Figure 4. Phylogenetic tree on the basis of nucleotide sequences of complete exon-9 of SLC11A1 gene of Vechur cattle and other mammalian species

Conclusion

The *SLC11A1* gene plays an important role in the innate immunity. The *SLC11A1* gene functions as metal ion transporter (divalent metals such as Fe^{2+} , Mn^{2+} , Zn^{2+}) involved in host defence against infections by acting as important cofactors for the production of toxic hydroxyl radicals. In the present study we identified a novel non synonymous single nucleotide variation in exon 9 of *SLC11A1* gene in Vechur cattle. We think that from this point the research on this field should be directed toward the identification of polymorphism in the coding and in the promoter regions of *SLC11A1* gene to further associate the eventual genotypes with phenotypes traits (resistant/susceptibility). The detected SNP can be used for further association analysis with disease incidence/resistance caused by intracellular pathogens in other cattle breeds.

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Karakterizacija eksona 9 membranskog transportnog proteina - SLC11A1 goveda vehur rase

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Rezime

A1 (SLC11A1) - membransko transportni A1 (*SLC11A1*) gen se povezuje sa prirodnom otpornošću na intracelularne patogene kao što su Brucella, Salmonella, Leishmania i Micobacterium u nekoliko vrsta uključujući goveda i igra kritičnu ulogu u eliminisanju patogena stvaranjem slobodnih radikala hidroksila. Cilj ovog istraživanja je bio da se ispita polimorfizam u eksonu 9 SLC11A1 gena u vehur goveda, vrsta patuljaste stoke u Indiji, poznata po otpornosti na bolesti. A 198 bp fragment, koji sadrži ekson 9 gena je pojačan korišćenjem polimeraze lančane reakcije (PCR). Analiza usaglašenost polimorfizam (SSCP) otkrila je dva različita trakasta obrasca. Novi, nesinonimi SNP (g.46C>T) sa dominantnim CC genotipom je takođe detektovan u goveda rase vehurke. Ovi rezultati pokazuju da postoji značajna genetska varijacija na SLC11A1 lokusi i dalja istraživanja ovih veza mogu da pomognu u razvoju testova genotipizacije zasnovanih na PCR metodama sa ciljem selekcije stoke sa boljim imunitetom na intracelularne patogene.

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