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Polymorphism in 3'UTR region of *Slc11a1* gene in Indian breeds of cattle

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ABSTRACT

The solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1 (*Slc11a1*) also called natural resistance-associated macrophage protein 1 gene (*Nramp1*) is a member of the large family of metal ion-transport proteins. It encodes a divalent cation (Fe+ & Mn+) transporter that localizes in the phagolysosome membrane in macrophages. *Slc11a1* gene plays a critical role in innate immunity favoring bacterial killing by macrophages in addition to its influence on adaptative immunity. Polymorphism at the 3' untranslated region (3'UTR) of *Slc11a1* gene is associated with natural resistance against brucellosis in cattle. Such polymorphisms are associated with variations in the number of GT repeats. This study aimed to discover polymorphism in the 3'UTR region of the *Slc11a1* gene in the Indian cattle breeds Nimari and Kenkatha. Polymerase Chain Reaction - Single Strand Conformation Polymorphism (PCR-SSCP) of 440 bp amplicon of *Slc11a1* gene revealed three common SSCP patterns in these breeds, which was also confirmed by detecting point mutation in sequences of these patterns. The study will augment the information available and be useful in further studies to determine the role of the *Slc11a1* gene in disease resistance and for the selection of brucellosis resistant animals.

Key words: cattle, *Slc11a1* gene, 3'UTR, polymerase chain reaction - single strand conformation polymorphism

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Introduction

The *Slc11a1* gene is a member of a large family of metal ion - transport proteins and was first recognized in mice as three loci *Lsh/Ity/Bcg*, that later proved to be the same *Slc11a1* and recently renamed as Slc11a1 (solute carrier family 11 member1) (VIDAL et al., 1993, 1995). The *Slc11a1* gene encodes a protein with 12 transmembrane domains that localizes in the phagolysosome membrane, particularly in macrophages (GRUENHEID et al., 1997). This protein has a pH dependent cation transport activity, acting as a transporter of divalent cations, such as iron (Fe2+) and manganese (Mn2+) from the lumen of the phagolysosome towards the cytosol, thus preventing acquisition of iron by intracellular pathogens (WYLLIE et al., 2002; FORBES and GROS, 2003). Interestingly, some experimental studies have suggested that the movement of cations may actually occur in the opposite direction, resulting in an increased concentration of iron into the phagolysosome, which may favor bacterial killing by generating oxygen intermediates through the Fenton reaction (ZWILLING et al., 1999; GOSWAMI et al., 2001). *Slc11a1* is implicated in pleiotropic effects, modulating adaptative immune response (BLACKWELL and SEARLE, 1999) as well as favoring stabilization of certain cytokine mRNA species (WYLLIE et al., 2002). In the mouse, *Slc11a1* gene confers resistance to several unrelated intracellular pathogens including *Mycobacterium* spp, *Salmonella* spp. and *Leishmania* spp. (VIDAL et al., 1995). In cattle, this gene has been associated with resistance against *Brucella abortus* infection (FENG et al., 1996; ADAMS and TEMPLETON, 1998; HORIN et al., 1999; BARTHEL et al., 2001), although there are contradictory findings, as KUMAR et al. (2005) and PAIXAO et al. (2007) did not detect any association between a 3' UTR polymorphism and resistance to brucellosis in cattle. Interestingly, BORRIELLO et al. (2006) and CAPPARELLI et al. (2006) studying the two *Slc11a1* alleles located in the 3'UTR of the water buffalo *Slc11a1* gene, found differences in the number of GT repeats and detected that the *Slc11a1*AA genotype confers susceptibility to *Brucella abortus* in water buffalo. MARTINEZ et al. (2008b) reported there are two allele (AA, AB and BB) in *Bos taurus*, *Bos indicus* cattle and in their animal crosses in the 3'UTR region of the *Slc11a1* gene, suggesting that the allele A may be associated with brucellosis resistance. MARTINEZ et al. (2008a) characterized the whole gene of *Slc11a1* except UTR and identified six new variants among a total of 11 single nucleotide mutations, of which five occurred in the coding sequence, one in the promoter region and five in introns in different breeds of *Bos taurus* and *Bos indicus*.

Indian cattle, also known as *zebu* cattle (*Bos indicus*), include 30 documented breeds of zebu cattle besides numerous populations found in various states of India yet to be characterized and defined (NIVSARKAR et al., 2000). The Indian zebu cattle have unique features such as adaptability to extreme climatic conditions and better resistance capabilities to withstand environmental stress and tropical disease. The Nimari breed is known as the "Biological Engine of Nimar", a breed originated from crossing Gir and Khillari breeds and its home tract is concentrated in the central part of the Khargone and

Barwani districts of Madhya Pradesh state, while Kenkatha is a draught cattle breed of the Panna, Chattarpur and Tikamgarh districts of Madhya Pradesh state and adjoining areas of Bundelkhand, comprising the Lalitpur, Hamirpur and Banda districts of Uttar Pradesh state.

Although intronic or UTR variation does not change the amino acid sequence of the protein, it may play a significant role in marker assisted selection. In livestock such variation in DNA may also be associated with economic traits, which are governed by many genes, each having a small effect (GELDERMAN, 1997). Some of the more frequently used methodologies for the identification of point mutations are Denaturing Gradient Gel Electrophoresis (DGGE) (FISHER and LERMAN, 1980; PATINO-GARCIA et al., 1999), Temperature Gradient Gel Electrophoresis (TGGE) (RIESNER et al., 1989) and Single Strand Conformation Polymorphism (SSCP) (ORITA et al., 1989a). SSCP is a simple and reliable technique, based on the assumption that changes in the nucleotide sequence of a polymerase chain reaction (PCR) product affect its single strand conformation. Molecules, differing by as little as a single base substitution, should have different conformers under non-denaturing conditions and migrate differently. Therefore, those differences can be detected as a shift in electrophoretic mobility (HAYASHI, 1991).

The aim of the present investigation was to identify the genetic variations in the 3' UTR of *Slc11a1* gene in the Nimari and Kenkatha breed of the *Bos indicus* cattle, using the PCR-SSCP technique and conformation of these mutations by sequencing.

Materials and methods

Blood samples (approximately 8 to 10 mL) were collected from 118 genetically unrelated cattle representative of the Nimari (n = 66) and Kenkatha (n = 52) breeds. The samples were collected from their respective home tracks. DNA was isolated by the method described by JOHN et al. (1991). Quality check and quantification were done by Nanodrop™ spectrophotometer and electrophoresis on 0.8% agarose gel. DNA concentration was determined and samples were diluted 10-30 times (approx. 30 ng/µl) with MiliQ water.

DNA amplification of the 3'UTR *Slc11a1* gene was achieved by PCR. Oligonucleotide primers (Forward- GGA AGC TGT GGG CCT TCA C and Reverse ATG CAG GAA GTC ATC GGC AG) as described by (BURGE et al., 1990) were used for the PCR amplification of the *Slc11a1* gene. Reaction was carried out in a final volume of 25 µl containing of 90 ng of genomic DNA, 1 X master mix (MBI Fermentas), 10 picomole of each forward and reverse primer and the remaining volume was adjusted with nuclease free water. PCR was carried out in a Bio-Rad thermal cycler. The thermal cycling profile was as follows: initial denaturation for 17 min. at 94 °C; followed by 30 cycles of denaturation at 94 °C for 1

min, annealing at 56 °C for 45 s and extension at 72 °C for 45 sec. The final extension was carried out for 10 min at 72 °C.

Each PCR product was diluted in a denaturing solution (95% formamide, 10 mM NaOH, 0.05% xylene cynol and 0.05% bromophenol blue, 20 mM EDTA), denatured at 94 °C for 5 min, immediately chilled on ice and resolved on 6% polyacrylamide gel. The electrophoresis was carried out in a Sequi-Gen GT nucleic acid sequencing cell (Bio-Rad) vertical electrophoresis unit using 1X TBE buffer at constant 5 W for SSCP analysis of all the fragments. Gel was silver-stained (SAMBROOK and RUSSELL, 2001) and dried on cellophane gel and scanned by GS-800 calibrated densitometer (Bio-Rad).

PCR amplicons that displayed a different pattern were selected for sequencing and were purified in low melting point agarose, following the method described by SAMBROOK and RUSSEL (2001). The concentration of the purified PCR product was determined and ligated using the InsT/Aclone™ PCR product cloning kit (MBI Fermentas) following the manufacturer's instructions. Ligated plasmids were transformed in DH5α and recombinant clones were selected by blue white screening. Recombinant plasmids were extracted and purified as per the method described by SAMBROOK and RUSSEL (2001). These purified recombinant plasmids were used as a template for cycle sequencing. Cycle sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Electrophoresis and data analysis was carried out on the ABI PRISM® 310 Genetic Analyzer using appropriate module, basecaller, dyeset/primer and matrix files.

Results

A 440 bp fragment of 3'UTR region of *Slc11a1* gene was successfully amplified in all samples. PCR - SSCP of the amplified fragment was performed to detect any mutation that might be present. Three types of SSCP band patterns were observed in both cattle breeds. The patterns were simple and consisted of only five bands. Pattern 1 consisted of band 1 and 4th; pattern 2 of bands 4 and 5 and pattern 3 of bands of bands 2 and 3 (Fig. 1). In Nimari the frequency observed was 0.636, 0.272 and 0.090 while it was 0.480, 0.384 and 0.134 in Kenkatha for Patterns P1, P2 and P3, respectively. The chi-square test revealed that the differences between observed frequencies of different patterns in Nimari and Kenkatha were non-significant at a 5% level of significance. The sample showing differential band patterns were subjected to cloning and sequencing. The sequences obtained for segment of *Slc11a1* have been submitted to the NCBI database (GQ923688, GQ923689 and GQ923690). The sequence analysis of the *Slc11a1* region revealed sequence variation. ClustalW analysis of this variant pattern of SSCP revealed in P1 T in place of C at position 140, G in place of A at position 259, T in place of C at 324 positions and T in place of G at position 345. In P2 there was pattern insertion of A at position 256, and in P3 C in place of T at position 119 (Fig. 2).

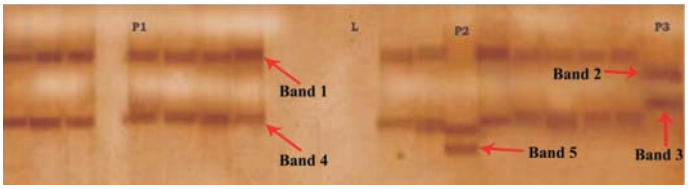

Fig. 1. Three SSCP patterns observed in 3' UTR region of *Slc11a1* gene

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Seq_2       GGAAGCTGTGGGCCTTCACTGAGTTTATGATCCTTCATCTCAGAAAAAGCTCTAAACAAC 60
Seq_3       ------------------------------------------------------------
seq_1       GGAAGCTGTGGGCCTTCACTGAGTTTATGATCCTTCATCTCAGAAAAAGCTCTAAACAAC 60

Seq_2       AAGCCCTCTGGGTGTATTTAAAGACAGCCGAGAAGGACTAAAAGATTCTACTTGGGAATC 120
Seq_3       ---CCCTCTGGGTGTATTTAAAGACAGCCGAGAAGGACTAAAAGATTCTACTTGGGAACC 57
seq_1       AAGCCCTCTGGGTGTATTTAAAGACAGCCGAGAAGGACTAAAAGATTCTACTTGGGAATC 120
               ********************************************************* *

Seq_2       TTCCTAGGAGGACACCCATCGCTCCCTCCACGCGTAAACGGGTCCCTCGATGCCTGTGAT 180
Seq_3       TTCCTAGGAGGACACCCATCGCTCCCTCCACGCGTAAACGGGTCCCTCGATGCCTGTGAT 117
seq_1       TTCCTAGGAGGACACCCATTGCTCCCTCCACGCGTAAACGGGTCCCTCGATGCCTGTGAT 180
            ****************** ****************************************

Seq_2       GGCATCTGTTGCTAATGGAGCATGTGAGGGTGAATCCCACAGAACCGAGTTGGGGAGCTG 240
Seq_3       GGCATCTGTTGCTAATGGAGCATGTGAGGGTGAATCCCACAGAACCGAGTTGGGGAGCTG 177
seq_1       GGCATCTGTTGCTAATGGAGCATGTGAGGGTGAATCCCACAGAACCGAGTTGGGGAGCTG 240
            ************************************************************

Seq_2       GGGGGGCAGTTGGCCAACCAAAGAATAGAAGAGCACACCAACCCAGTCCCCAGGGCCTGG 300
Seq_3       GGGGGGCAGTTGGCCA-CCAAAGAATAGAAGAGCACACCAACCCAGTCCCCAGGGCCTGG 236
seq_1       GGGGGGCAGTTGGCCA-CCAGAGAATAGAAGAGCACACCAACCCAGTCCCCAGGGCCTGG 299
            **************** *** ************************************

Seq_2       GAAGAAGAGAGGCTTTTACCACGCTCCCTGGCAGGAGGCTGGGGGGTGTTTTTCCTGAAA 360
Seq_3       GAAGAAGAGAGGCTTTTACCACGCTCCCTGGCAGGAGGCTGGGGGGTGTTTTTCCTGAAA 296
seq_1       GAAGAAGAGAGGCTTTTACCACGTTCCCTGGCAGGAGGCTGGGGTGTGTTTTTCCTGAAA 359
            ********************** ******************** ***************

Seq_2       TCTCTGCAGGGCCCTATAAGAGGCTGTGGGGATGATGAGCAGAAGAAGAGGGTGCTGGGT 420
Seq_3       TCTCTGCAGGGCCCTATAAGAGGCTGTGGGGATGATGAGCAGAAGAAGAGGGTGCTGGGT 356
seq_1       TCTCTGCAGGGCCCTATAAGAGGCTGTGGGGATGATGAGCAGAAGAAGAGGGTGCTGGGT 419
            ************************************************************

Seq_2       ACATGCAGGAAGTCATCCAGAG 442
Seq_3       ACATGCAGGAAGTCATC----- 373
seq_1       ACATGCAGGAAGTCATCGGCAG 441
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Fig. 2. ClustalW report: Seq_1 representing Pattern 1 (P1), Seq _2 representing Pattern 2)P2), Seq_3 representing Pattern 3 (P83)

Discussion

Single Strand Conformation Polymorphism offers a simple, inexpensive and sensitive method for detecting whether or not DNA fragments are identical in sequence, and so can greatly reduce the amount of sequencing necessary (ORITA et al., 1989a; ORITA et al., 1989b; HAYASHI, 1991; HAYASHI, 1992; HAYASHI and YANDELL, 1993). Several authors have pointed out that PCR-SSCP analysis is an appropriate, reliable and reproducible tool for detection of structural gene polymorphism that is primarily due to point mutations (NEIBERGS et al., 1993; SHEFFIELD et al., 1993; BARROSO et al., 1998; KUMAR et al., 2006). In fact, NEIBERGS et al. (1993) stated that PCR-SSCP analysis is the technique of choice when screening point mutations and minor deletions within a given fragment, although it is necessary to optimize conditions for each case. The results of the present study provide the first evidence of the genetic variability of the 3'UTR *Slc11a1*gene within the Indian Nimari and Kenkatha cattle breeds. Apart from variability in the 3'UTR region of the *Slc11a1* gene, sequencing of this region showed these sequences are novel. The results obtained in the present study were different from the findings of the other studies reported by KUMAR et al. (2005), BORRIELLO et al. (2006), PAIXAO et al. (2006, 2007) and MARTINEZ et al. (2008b) because the location of the primer used in the present study was different from those used by them. The regions of attachment of the primer in all cases are in the 3'UTR, but in the present study the attachment site of the primer is in different locations in the 3'UTR. Polymorphism is generally found in the coding and noncoding region of Nucleic acid, but in this 3 types of SSCP patterns clearly showed that polymorphism can also present in the 3'UTR region of DNA and this polymorphic site may act as an enhancer or silencer for the defenses mechanism of brucellosis. This point mutation can be used further as SNP markers which could be helpful to breeders for future association studies, selecting superior germplasm and conservation strategies. The study will augment the information available and will be useful in further studies to determine the role of the *Slc11a1* gene in disease resistance and for selection of brucellosis resistant animals, it may be useful for establishing a possible association with productive parameters.

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SAŽETAK

Među proteinima prijenosnicima metalnih iona, familija 11 i njezin član 1 (*Slc11a1*) zauzimaju značajno mjesto zbog činjenice da se povezuju s prirodnom imunošću. Na temelju toga za *Slc11a1* učestalo se rabi i naziv gen za makrofagni protein 1 (*Nramp1*). Gen kodira protein-prijenosnik dvovalentnog kationa (Fe+ i Mn+) koji je smješten u fagolizosomu membrane makrofaga. Gen *Slc11a1* ima važnu ulogu u nespecifičnoj imunosti, prvenstveno pri ubijanju bakterija makrofagima, no pretpostavlja se njegova dodatna uloga u specifičnoj imunosti. Polimorfizam u 3' nekodirajućoj regiji (3'UTR) gena *Slc11a1* osniva se na različitom broju GT ponavljanja i dovodi u vezu s prirodnom otpornošću goveda prema brucelozi. Ovim istraživanjem želi se utvrditi polimorfizam u 3'UTR regiji gena *Slc11a1* u indijskih pasmina goveda, nimari i kenkatha. Primjenom lančane reakcije polimerazom odnosno analizom polimorfizma jednolančane konformacije amplikona s 440 baznih parova gena *Slc11a1*, u promatranih pasmina utvrđene su tri varijante jednolančane konformacije. Navedeno je također potvrđeno opažanjem točkastih mutacija u sekvencijama tih varijanata. Istraživanje će pridonijeti količini informacija iz predmetnog područja te biti korisno za buduća istraživanja koja imaju zadatak utvrditi ulogu gena *Slc11a1* u otpornost na bolesti kao i u odabiranju životinja otpornih na brucelozu.

Ključne riječi: govedo, gen *Slc11a1*, 3'UTR, lančana reakcija polimerazom, polimorfizam jednolančane konformacije