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DNA polymorphism of 3' UTR of Nramp1 gene in Malvi breed of cattle

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Abstract

The natural resistance-associated macrophage protein 1 gene (Nramp1), which is a member of large family of metal ion–transport protein. Nramp1 gene plays a critical role in innate immunity favoring bacterial killing by macrophages in addition to its influence on adaptive immunity. The aim of the present investigation was to identify the genetic variations in the 3'UTR (Untranslated region) of Nramp1 gene in the Malvi breed (Bos indicus) cattle, using the technique PCR-SSCP and by sequencing. PCR-SSCP (Single Strand conformational polymorphism) of 440 bp amplicon of Nramp1 gene revealed three common SSCP patterns in Malvi breed. A total of 3 SSCP patterns viz Pattern I, Pattern II and Pattern III were observed with frequency of 0.361, 0.426 and 0.213 respectively. The patterns variations were confirmed by cloning and sequencing, which showed total 6 mutations in 3 patterns.
Keywords: Malvi cattle, Nramp1, Polymorphism, SSCP, Sequencing

Introduction

The Indian cattle, also known as zebu cattle (Bos indicus), there are 30 documented breeds of zebu cattle besides, numerous populations found in various states of India are yet to be characterized and defined (Nivsarkar *et al*., 2000). The Indian zebu cattle have unique features like adaptability to extreme climatic conditions and better resistance capabilities to withstand environmental stress and tropical disease. Malvi, a well-known draft purpose breed belongs to Malva region of Madhya Pradesh state and also from western adjoining parts of Rajasthan state.

The Nramp1 gene encodes a protein with 12 transmembrane domains that localizes in the phagolysosome membrane, particularly in macrophages (Gruenheid *et al*., 1997). The Nramp1 gene is a member of a large family of genes coding for metal ion-transporting proteins and first time identified in the mouse as three loci Lsh/Ity/Bcg, that latter proved to be the same Nramp1 and recently renamed as Slc11a1 (solute carrier family 11 member1) (Vidal *et al*., 1995). This protein has a pH dependent cation transport activity, acting as a transporter of divalent cations such as iron (Fe^{2+}) and manganese (Mn^{2+}) from the lumen of the phagolysosome towards the cytosol, thus preventing acquisition of iron by intracellular pathogens (Wyllie *et al*., 2002). Nramp1 is implicated in pleiotropic effects, modulating adaptive immune response as well as favoring stabilization of certain cytokine mRNA species (Wyllie *et al*., 2002). Nramp1 confers innate resistance to intracellular pathogens; it has been proposed that the product of Nramp1 may limit microbial replication in the phagosome by subtracting critical nutrients to invading microbes (Gruenheid *et al*., 2000).

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 (Orita *et al*., 1989). 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). By using this technique the aim of the present investigation was to identify the genetic variations in the 3'UTR of Nramp1 gene in the Malvi breed of cattle, using PCR-SSCP and further confirmed by cloning and sequencing.

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Materials and methods

Total of 61 blood samples of unrelated males and females from Malvi (Ujjain and Shajapur districts of MP) were collected from breeding tract and Government cattle breeding farms. The DNA was isolated by the method as described by John et al. (1991). Quality check and quantification was done by gel electrophoresis (0.8% agarose) and NanodropTM spectrophotometer at optical density (OD) 260nm/280nm respectively. The DNA concentration was determined and samples were diluted 10-30 times (approx. 30 ng/µl) with MiliQ water.

DNA amplification of the 3'UTR Nramp1 gene was achieved by PCR. Oligonucleotides primers (Forward-GGAAGCTGTGGGCCTTCAC and Reverse ATGCAGGAAGTCATCGGCAG) as described by Burge et al. (2004) were used for the PCR amplification. Reactions were carried out in a final volume of 25 µl containing of 90-100ng of genomic DNA, 1 X master mix (MBI Fermentas), 10 picomole of each forward and reverse primer and remaining volume was adjusted with nuclease free water. PCR was carried out in a Biorad 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 45s. 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 then scanned by GS-800 calibrated densitometer (Biorad).

PCR amplicons that displayed 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 template for cycle sequencing. Cycle sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Apllied Biosystems, USA). Electrophoresis and data analysis was carried out on the ABI PRISM® 310 Genetic Analyzer.

Results

A 440 bp fragment of 3' UTR region of Nramp1 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; the patterns were simple and consisted of only three bands. The frequency observed was 0.361, 0.426 and 0.213 respectively for pattern 1 to 3 respectively. The chi-square test revealed that difference between observed frequencies of different patterns in Malvi was non-significant at 5% level of significance. The sample showing differential band patterns were subjected to cloning and sequencing. The sequences obtained for segment of Nramp1 have been submitted to NCBI database, the accession numbers are FJ236546 (pattern I), FJ236545 (pattern II) and FJ236548 (pattern III). The ClustalW analysis of sequences of Nramp1 region revealed sequence variation as shown in table1 and table 2.

Discussion

The result of present study provides the first evidence of genetic variability of the 3'UTR Nramp1 gene within the Indian Malvi breed. Apart from variability in the 3'UTR region of Nramp1 gene sequencing of this region showed these sequences are novel. The present study result revealed that all three sequences showed sequence variation when compared with each other.

The results obtained in the present study were different with the findings ofother studies (Kumar *et al*., 2005, Borrielo *et al*., 2006, Paixao *et al*., 2006, 2007 and Martinez *et al*., 2008), because the location of the primer used in the present study was

Table 1. Nucleotide change in the sequences

Base number	Pattem I (FJ236546)	Pattem II (FJ236545)	Pattem III (FJ236548)
29	C	C	T
140	C	T	C
257	A	Deletion	Deletion
261	A	G	A
323	C	T	C
344	G	T	G

Table 2. Clustal W report

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FJ236546  ----------------ACTGAGTTTATGATCCTTCATCTCAGAAAAAGCTCTAAACAAC 43
FJ236548  ----------------------------TCTTCATCTCAGAAAAAGCTCTAAACAAC 29
FJ236545  GGAAGCTGTGGGCCTTCACTGAGTTTATGATCCTTCATCTCAGAAAAAGCTCTAAACAAC 60
                                        ****************************

FJ236546  AAGCCCTCTGGGTGTATTTAAAGACAGCCGAGAAGGACTAAAAGATTCTACTTGGGAATC 103
FJ236548  AAGCCCTCTGGGTGTATTTAAAGACAGCCGAGAAGGACTAAAAGATTCTACTTGGGAATC 89
FJ236545  AAGCCCTCTGGGTGTATTTAAAGACAGCCGAGAAGGACTAAAAGATTCTACTTGGGAATC 120
          ************************************************************

FJ236546  TTCCTAGGAGGACACCCATCGCTCCCTCCACGCGTAAACGGGTCCCTCGATGCCTGTGAT 163
FJ236548  TTCCTAGGAGGACACCCATCGCTCCCTCCACGCGTAAACGGGTCCCTCGATGCCTGTGAT 149
FJ236545  TTCCTAGGAGGACACCCATTGCTCCCTCCACGCGTAAACGGGTCCCTCGATGCCTGTGAT 180
          ******************* ****************************************

FJ236546  GGCATCTGTTGCTAATGGAGCATGTGAGGGTGAATCCCACAGAACCGAGTTGGGGAGCTG 223
FJ236548  GGCATCTGTTGCTAATGGAGCATGTGAGGGTGAATCCCACAGAACCGAGTTGGGGAGCTG 209
FJ236545  GGCATCTGTTGCTAATGGAGCATGTGAGGGTGAATCCCACAGAACCGAGTTGGGGAGCTG 240
          ************************************************************

FJ236546  GGGGGGCAGTTGGCCAACCAAAGAATAGAAGAGCACACCAACCCAGTCCCCAGGGCCTGG 283
FJ236548  GGGGGGCAGTTGGCCA-CCAAAGAATAGAAGAGCACACCAACCCAGTCCCCAGGGCCTGG 268
FJ236545  GGGGGGCAGTTGGCCA-CCAGAGAATAGAAGAGCACACCAACCCAGTCCCCAGGGCCTGG 299
          **************** *** ********************************** ****

FJ236546  GAAGAAGAGAGGCTTTTACCACGCTCCCTGGCAGGAGGCTGGGGGGTGTTTTTCCTGAAA 343
FJ236548  GAAGAAGAGAGGCTTTTACCACGCTCCCTGGCAGGAGGCTGGGGGGTGTTTTTCCTGAAA 328
FJ236545  GAAGAAGAGAGGCTTTTACCACGTTCCCTGGCAGGAGGCTGGGGTGTGTTTTTCCTGAAA 359
          ********************** ******************** ****************

FJ236546  TCTCTGCAGGGCCCTATAAGAGGCTGTGGGGATGATGAGCAGAAGAAGAGGGTGCTGGGT 403
FJ236548  TCTCTGCAGGGCCCTATAAGAGGCTGTGGGGATGATGAGCAGAAGAAGAGGGTGCTGGGT 388
FJ236545  TCTCTGCAGGGCCCTATAAGAGGCTGTGGGGATGATGAGCAGAAGAAGAGGGTGCTGGGT 419
          ************************************************************

FJ236546  ACATGCAGGAAGTCATCCAGAG 425
FJ236548  ACATGCAGGAAGTCATC----- 405
FJ236545  ACATGCAGGAAGTCATCGGCAG 441
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different from those used by them. The region of attachment of the primer in all the cases were in the 3'UTR but in the present study attachment site of primer was in different locations in the 3'UTR. Polymorphism is generally found in the coding and non-coding region of nucleic acid, but in this study, the 3 type of SSCP patterns clearly showed that polymorphism can also present in 3'UTR region of DNA and this polymorphic site may act as enhancer or silencer for the defenses mechanism of brucellosis. This point mutation can further be used 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 and will be useful in further studies to determine the role of Nramp1 gene in the disease resistance and for the selection of brucellosis resistant animal and may be useful for establishing a possible association with productive parameters.

Conclusion:

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). The study will augment the information and will be useful in further studies to determine the role of Nramp1 gene in the disease resistance and for the selection of brucellosis resistant animal may be useful for establishing a possible association

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