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Analysis of genetic variations of complete TM4 of buffalo (*Bubalus bubalis*) *Slc11A1* gene

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To explore polymorphisms at exon V–VII, encompassing complete TM4 and part of TM3 and TM5 of buffalo *SLC11A1* gene, 150 animals belonging to three breeds of buffalo (*Bubalus bubalis*) viz., Murrah, Surti and Mehsana were genotyped by PCR-RFLP using five restriction enzymes (REs) (*Alu* I, *Taq* I, *Rsa* I, *Sac* I and *Bse* GI). PCR–RFLP revealed monomorphic restriction pattern among the three buffalo breeds. Although, sequence comparison revealed no variations at nucleotide level among the buffalo breeds under study, however, two synonymous substitutions at amino acid position 145 (A→C transversion) and 151 (T→C transition) of TM3 were observed compared to cattle sequence (NM-174652). No changes in TM4 and synonymous substitutions at TM3 reflect the conserve nature of this region specially TM4 in buffalo. Future studies may be directed to explore polymorphisms throughout the entire buffalo *SLC11A1* gene and to ascertain their suitability as potential genetic marker for resistance against various diseases.

Keywords: NRAMP1; polymorphism; PCR–RFLP; buffalo

Introduction

The solute carrier family 11A member 1 (*SLC11A1*) gene (formerly *NRAMP1*), coding for an iron/divalent cation transporter (Blackwell et al. 2003), has been reported to confer resistance or susceptibility to a number of antigenically different intracellular pathogens namely *Salmonella typhimurium*, *Leishmania donovani* and *Mycobacterium bovis* in mice (Vidal et al. 1993). A G169D point mutation of *SLC11A1* gene located within the fourth transmembrane domain (TM4) is invariably associated with resistance and susceptible phenotypes, respectively. Although, the point mutation (G169D) has never been documented in cattle or other mammalian species, however, several polymorphisms within coding and non coding regions of *SLC11A1* gene have been associated with resistance/susceptibility to infectious agents and to autoimmune disorders in human (Blackwell et al. 2003; Awomoyi 2007), *Brucella abortus* in water buffalo (Capparelli et al. 2007; Ganguly et al. 2008), *Rhodococcus equi* pneumonia in horses (Halbert et al. 2006), leishmaniasis in dog (Sanchez-Robert et al. 2005) and *Salmonella choleraesuis* in pig (Tuggle et al. 2005).

Considering the immense importance of this gene towards innate resistance against various diseases and the economic importance of buffalo in Indian context, the present study was undertaken with the objective of identifying and comparing polymorphisms in and around TM4 of *SLC11A1* gene in different buffalo breeds using PCR–RFLP as well as nucleotide sequencing and to characterise the polymorphic region.

Materials and methods

In the present study a total of 150 animals belonging to three different breeds of riverine buffalo (*Bubalus bubalis*) viz. Murrah ($n = 60$), Mehsana ($n = 50$) and Surti ($n = 40$) were included. Genomic DNA was isolated from the venous blood using standard phenol chloroform extraction method (Sambrook et al. 1989). A fragment of 954 bp, encompassing the complete TM4, was amplified using PCR (Table 1). PCR was carried out with a final reaction volume of 50 µl containing 100 ng of template DNA, 1 × PCR assay buffer, 2.0 mM of Mg^{2+}, 200 µM dNTPs, 10 pM of each primer and 1.0U of *Taq* DNA polymerase. To rule out PCR carryover, a negative control without template (genomic DNA) was always included. After amplification, PCR products and DNA molecular weight ladder were electrophoresed on agarose gels (1%) containing ethidium bromide, in order to confirm the anticipated fragment sizes.

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Table 1. Descriptions of the primers, amplified region, product length and RE used to explore polymorphism of *Slc11A1* gene in buffalo.

Primers name and sequence	Region amplified		Cyclic profile	Amplicon length	RE used	Restriction fragment lengths
	Exons	TM domains				
NRE5F 5′ TCCGACATGCAGGA AGTCAT3′	V (partial), VI (complete) and VII (partial)	3 (partial), 4 (complete) and 5 (partial)	94°C 5 min (ID)		Alu I	541, 255, 79, 51 and 28 bp
			35 cycles		Taq I	542 and 412 bp
NRE7R5′GCCGAAGGTCAAGGC CATTATGG3′			94°C 40 sec		Rsa I	559, 334 and 61 bp
			60°C 40 sec		Bse G I	954 bp only
			72°C 1 min 30 sec	954 bp	Sac I	422, 385 and 147 bp
			Final extension 72°C 10 min			
			4°C hold			

Animals were genotyped by PCR–RFLP using five REs (*Alu* I, *Taq* I, *Rsa* I, *Sac* I and *Bse* GI). The restriction digestion was carried out in 25 µl final volume following manufacturer instructions overnight in a water bath at 37°C except *Taq* I (65°C) and *Bse* G I (55°C). Briefly, 10U RE was used for every 15 µl PCR product. Restriction patterns were analysed by agarose gel (2%) electrophoresis in 1 × Tris-acetate-EDTA (TAE) buffer for 2–3 hours at 5 V/cm and visualised under UV-transilluminator. Corresponding amplicons of representative random samples of each breed were sequenced. Partial exons of submitted sequences were compared with the corresponding region of available cDNA and mRNA sequences of other species. Sequence divergence and phylogenetic tree was constructed.

Results and discussion

The region was chosen in the present investigation because of the fact that TM4, within exon V–VII, harbour one important mutation (G169D) in murine Nramp 1 protein, which makes individual susceptible towards a number of intracellular pathogens. PCR-generated amplicons were also included part of preceding and succeeding TM domain (Table 1). In this study no polymorphism was detected within animals across the buffalo breeds. Single PCR–RFLP pattern was resulted in all the animals with respect to each RE utilised (Figure 1, Table 1). Fragments <30 bp (in *Alu* I digestion) could not be resolved properly in agarose gel due to its small size.

Figure 1. PCR–RFLP patterns of buffalo *SLC11A1* gene exon V–VII. Lane 1–2: *Alu* I digestion pattern (541, 255, 79 and 51 bp); Lane 3–4: *Taq* I digestion pattern (542 and 414 bp); Lane 5: Uncut (954 bp); Lane 6: 100 bp DNA ladder; Lane 7–8: *Rsa* I digestion pattern (559, 334 and 61 bp); Lane 9–10: *Sac* I digestion pattern (954 bp) and Lane 11–12: *Bse* GI digestion pattern (422, 385 and 147 bp).

Several studies have been initiated to explore new DNA polymorphisms of *Slc11A1* gene and to ascertain their association with the host resistance/susceptibility against different intracellular pathogens in human (Blackwell et al. 2003; Awomoyi 2007), cattle (Kumar et al. 2005; Martinez et al. 2008), buffalo (Capparelli et al. 2007; Ganguly et al. 2008), horse (Halbert et al. 2006) and pig (Tuggle et al. 2005). However, lack of polymorphism at TM4 of *Slc11A1* gene among Murrah, Surti and Mehsana breeds once again revealed that buffalo is mostly monomorphic in comparison to other species especially to cattle as observed in Kappa casein (Pipalia et al. 2001), IGFBP-3 (Kumar et al. 2006), DGAT1 and ABCG2 (Tantia et al. 2006) and other important gene polymorphism studies. In contrast to the above finding, we observed *Alu* I and *Taq* I polymorphisms in intron 6 and intron 5 of *SLC11A1* gene and their association with brucellosis resistance in Indian zebu (*Bos indicus*) and crossbred (*Bos indicus* X *Bos taurus*) cattle was studied (Kumar et al. 2011).

Sequencing results from all the three buffalo breeds in the present study confirmed the amplicon length of 954 bp. Comparison of nucleotide sequences (AY707989, AY860618 and AY860620) demonstrated no differences among buffalo breeds. These sequences are the first report in Indian Buffaloes. Comparative study of Murrah/Mehsana/Surti *SLC11A1* coding sequences with corresponding region of available buffalo (U27105) and cattle (NM-174652) cDNA sequences at nucleotide and amino acid level was carried out (Table 2). Two synonymous substitutions at TM3 were observed in all buffalo sequences, one at amino acid position 145 (A→C transversion) and another at amino acid position 151 (T→C transition), respective to available cattle sequence (NM-174652) (Table 2). No changes in TM4 and synonymous substitutions at TM3 reflect the conserve nature of this region specially TM4. Synonymous mutations do not alter coding sequences, and therefore they are not expected to change the physical properties, expression and function of the protein concern. However, recently, Nackley et al. (2006) and Capon et al. (2004) have provided evidence that synonymous single-nucleotide polymorphisms (SNPs) can effect protein expression and thereby function by modulating mRNA stability. Moreover, a recent finding claimed that a 'silent' polymorphism do change substrate specificity (Kimchi-Sarfaty et al. 2007). Therefore, it is difficult at present to rule out any possible role of synonymous substitution at buffalo TM3 and mRNA stability of *SLC11A1* gene compare to cattle.

Sequence analysis of all the three buffalo breeds revealed the presence of a Gly at exon VI, which is the same amino acid of the murine *NRAMP1*-resistant phenotype at amino acid position 169 of TM4. As expected, cattle and buffalo were found to form single monogenic group as compared to other species (Figure 2). The species next to these two species were sheep, followed by red deer, bison, pig, human and chicken. Partial exon V, complete exon VI and partial exon VII resulted a 64 amino acid sequence. It showed 100% similarity with available buffalo (U27105) and cattle (NM-174652) sequences. The amino acid sequence similarity of sheep, red deer, bison, pig human and chicken with Murrah sequence was found to be approximately 98.4%, 98.4%, 96.9%, 95.3%, 90.6% and 93.8%, respectively.

In conclusion, although analysis of TM4 of *SLC11A1* gene by PCR–RFLP and automated DNA sequencing revealed monomorphic pattern and no variation in coding region among the three riverine buffalo breeds, however, two synonymous substitutions at amino acid position 145 (A→C transversion) and 151 (T→C transition) of TM3 were observed in buffaloes as compared to *B. taurus* (NM_174652).

Table 2. Nucleotide changes (with or without amino acid changes) in Buffalo (*Bubalus bubalis* and *Bubalus arnee bubalis*) compared to *B. taurus* sequence (NM_174652).

Position[a] Amino acid	Nucleotide and amino acid changes	Species[b]	Exon	Features[c]
Partial exon V, complete exon VI and partial exon VII				
145	Ser (TC**A**) → Ser (TC**C**)	*Bubalus bubalis*[d]	V	TM3
151	Ile (AT**T**) → Ile(AT**C**)	*Bubalus bubalis*[d], *Bubalus arnee bubalis*	V	TM3
193	Glu (GA**A**) → Glu (GA**G**)	*Bubalus arnee bubalis*	VII	TM5

Note: TM, transmembrane domain.
Bubalus bubalis[d]: Murrah/ Mehsana/Surti buffalo sequence (AY707989/AY860618/AY860620).
[a]Position of the residue is given with respect to *Bos taurus* NRAMP1 protein.
[b]Changes given with respect to *Bos Taurus*;.
[c]Adapted from Coussens et al. (2004).

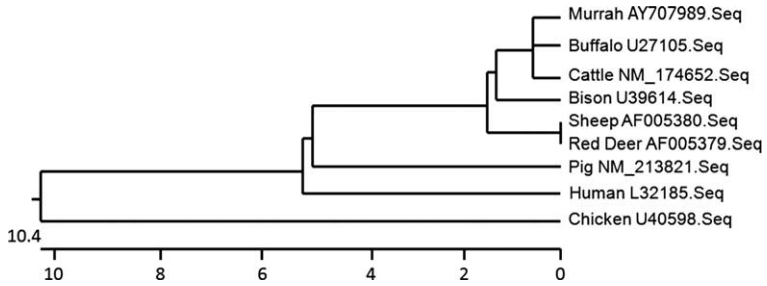

Figure 2. Phylogram based on nucleotide coding sequences from exon V–VII of *SLC11A1* gene.

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